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Cyclophosphamide is the most widely used drug in the treatment of breast cancer. Unfortunately, emergence of drug-resistant tumor cell populations limits its usefulness. Class-1 and class-3 aldehyde dehydrogenases (ALDH-1 and ALDH-3, respectively) have been shown to catalyze the detoxification of cyclophosphamide and other oxazaphosphorines. Predictably, then, relatively elevated levels of ALDH-1 and ALDH-3 have been shown to account for resistance to these agents in several cultured breast and other tumor cell models. It follows that cellular resistance to these agents on the part of clinical breast cancers could be due to overexpression of ALDH-1 and/or ALDH-3. Our finding that elevated levels of ALDH-1 and ALDH-3 are present in some primary and metastatic breast tumor tissues supports this notion. Although nearly identical, tumor cell ALDH-3 appears to be different from normal cell ALDH-3. A tumor-specific ALDH-3 would have diagnostic potential. Tumor cell ALDH-3 was found to be more sensitive to inhibition by each of five chlorpropamide analogs than was normal cell ALDH-3 suggesting that selective sensitization of tumor cells to cyclophosphamide and other oxazaphosphorines may be possible when tumor cell insensitivity to these drugs is due to high levels of ALDH-3. Xenobiotics that are abundantly present in the diet/environment, e.g., methylcholanthrene and catechol, rapidly, coordinately and reversibly induced ALDH-3 and other drug metabolizing enzymes in cultured breast and other cancer cell models, thus, rapidly effecting reversible multienzyme-mediated multidrug resistance/collateral sensitivity to cyclophosphamide and certain other anticancer drugs. Consumption of dietary substances rich in such inducers, e.g., coffee and broccoli, resulted in elevated levels of ALDH-3 and other enzymes in human saliva. Thus, the environment and diet may influence the therapeutic efficacy of cyclophosphamide and certain other anticancer drugs. If so, these factors need to be considered when attempting to choose the most suitable agent for clinical use.

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INTRODUCTION

Cyclophosphamide, mafosfamide and 4-hydroperoxycyclophosphamide are antineoplastic agents collectively referred to as oxazaphosphorines [Sladek, 1994]. Each of these is a prodrug, i.e., per se, without cytotoxic activity. Salient features of the metabolic activation of oxazaphosphorines are presented in Figure 1. Oxazaphosphorines are clinically effective; they play a lead role in the treatment of breast cancer until resistant subpopulations become the dominant population. An understanding of how resistance to these agents is effected would likely to be of value because measures may then become apparent as to how to reverse, and/or prevent it. It is this understanding which is the overall objective of our first-generation investigations.

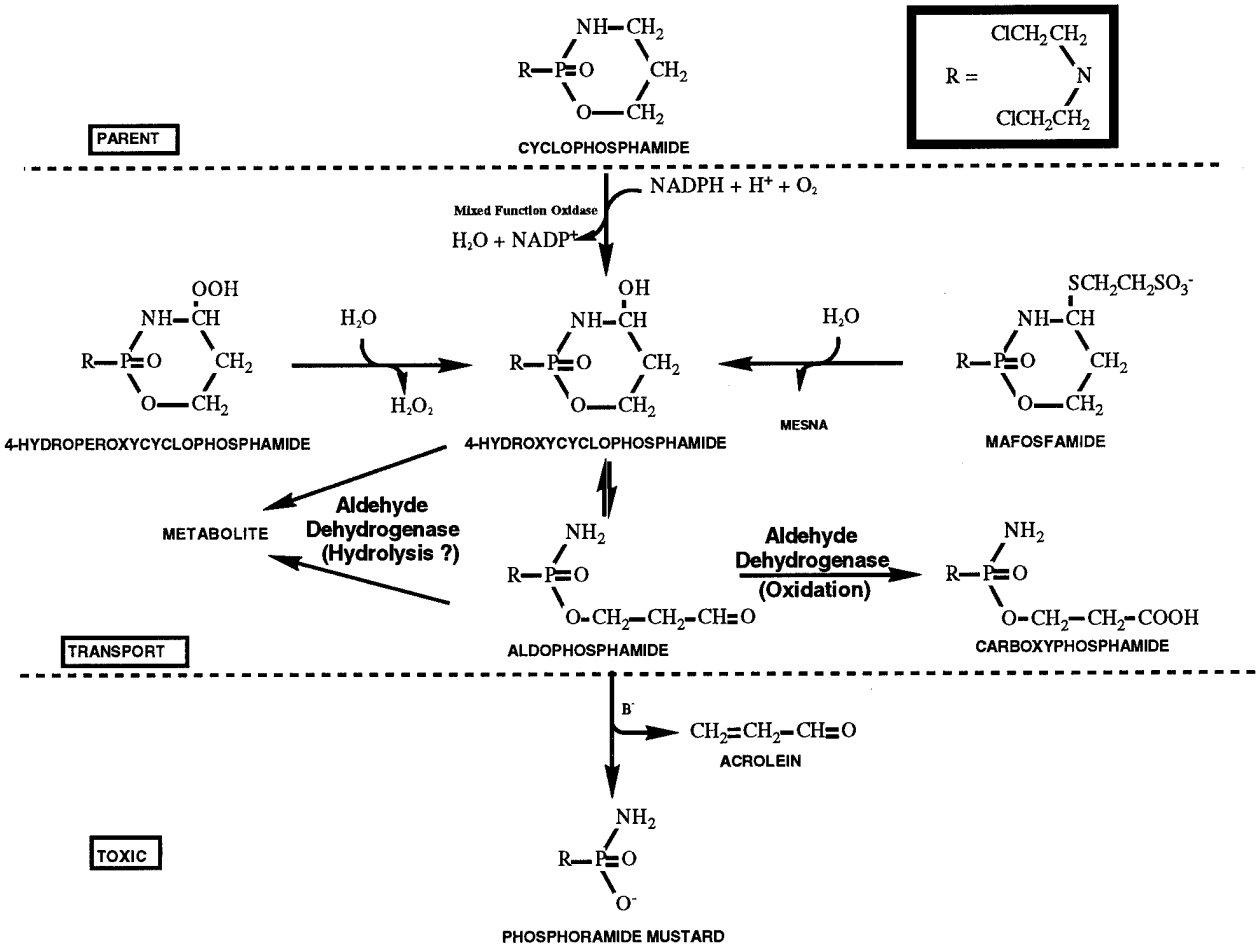


Figure 1. Salient features of oxazaphosphorine metabolism. The prodrugs, cyclophosphamide, mafosfamide and 4-hydroperoxycyclophosphamide, each give rise to 4-hydroxycyclophosphamide which exists in equilibrium with its ring-opened tautomer, aldophosphamide. 4-Hydroxycyclophosphamide and aldophosphamide are, themselves, also without cytotoxic activity. However, aldophosphamide gives rise to acrolein and phosphoramide mustard, each of which is cytotoxic; the latter effects the bulk of the therapeutic action effected by the oxazaphosphorines [Sladek, 1994]. Alternatively, aldophosphamide can be further oxidized to carboxyphosphamide by certain aldehyde dehydrogenases [Manthey et al., 1990; Dockham et al., 1992; Sreerama and Sladek, 1993a, 1994; Sladek, 1994]. Carboxyphosphamide is without cytotoxic activity nor does it give rise to a cytotoxic metabolite. Aldehyde dehydrogenase-catalyzed oxidation of aldophosphamide to carboxyphosphamide is, therefore, properly viewed as an enzyme-catalyzed detoxification of the oxazaphosphorines. Aldehyde dehydrogenase-catalyzed hydrolysis of 4-hydroxycyclophosphamide and/or aldophosphamide to an inactive metabolite is also shown but is only a possibility, i.e., it is yet to be demonstrated.

Most pertinent to these investigations is the irreversible detoxification that occurs when NAD(P)-dependent aldehyde dehydrogenases catalyze the oxidation of a pivotal metabolite, viz., aldophosphamide, to carboxyphosphamide, Figure 1. Human class-1, -2 and -3 aldehyde dehydrogenases, viz., ALDH-1, ALDH-2 and ALDH-3, respectively, as well as succinic semialdehyde dehydrogenase, all catalyze the oxidation of aldophosphamide to carboxyphosphamide, but not equally well [Dockham et al., 1992; Sladek, 1993, 1994; Sreerama and Sladek, 1993a, 1994].

Aldehyde dehydrogenases are bifunctional enzymes in that they catalyze not only the oxidation of aldehydes, but also the hydrolysis of ester bonds. Several such bonds are present in 4-hydroxycyclophosphamide and aldophosphamide, Figure 2. Whether aldehyde dehydrogenases catalyze the hydrolysis of either of these intermediates to an irreversibly inactive metabolite is not known.

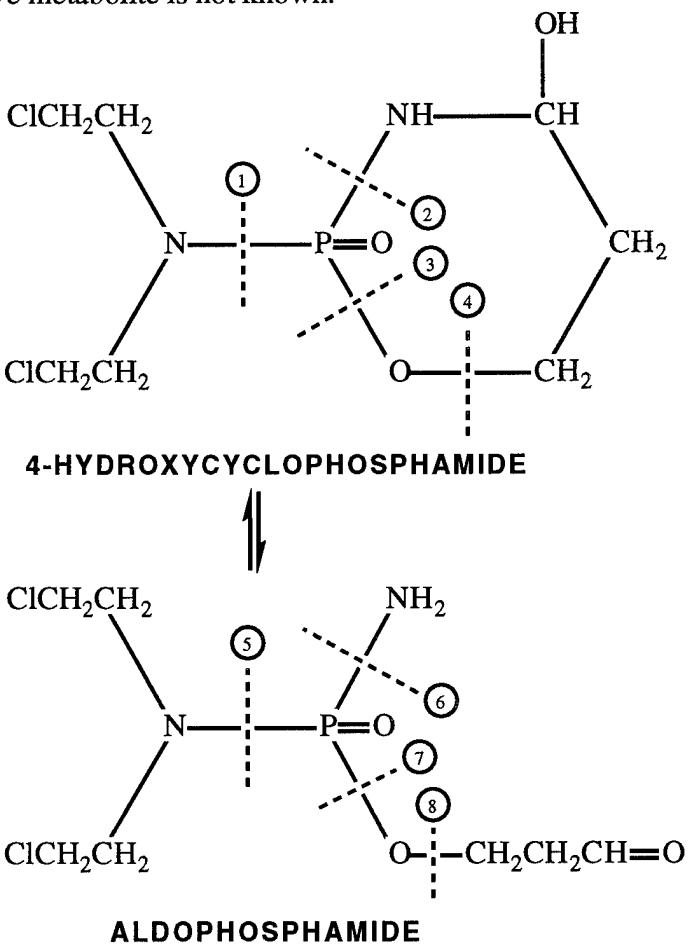


Figure 2. Aldehyde dehydrogenase-catalyzed hydrolysis of 4-hydroxycyclophosphamide and aldophosphamide: possibilities. Hydrolysis of 4-hydroxycyclophosphamide at (1) and aldophosphamide at (5) would give rise to bis-(2-chloroethyl)-amine. Thus, hydrolysis at (1) or (5) cannot account for oxazaphosphorine detoxification, because bis-(2-chloroethyl)-amine is more cytotoxic than are the prodrugs, e.g., mafosfamide and 4-hydroperoxycyclophosphamide, from whence it would originate [Sreerama and Sladek, 1993a; Sladek, 1994]. Hydrolysis of aldophosphamide at (7) and (8) would give rise to phosphoramide mustard. Thus, hydrolysis at (7) and (8) cannot account for the oxazaphosphorine detoxification, because phosphoramide mustard is more cytotoxic than are the prodrugs, e.g., mafosfamide, 4-hydroperoxycyclophosphamide, from whence it would originate [Sreerama and Sladek, 1993a, 1994; Sladek, 1994].

Using cultured human breast adenocarcinoma MCF-7/0 cells and two oxazaphosphorine-resistant sublines derived therefrom, viz., MCF-7/OAP (stable resistance achieved by growing the parent MCF-7/0 cells in the presence of continuously increasing concentrations of 4-hydroperoxycyclophosphamide for many months [Frei et al., 1988]) and MCF-7/PAH (transient resistance achieved by growing the parent MCF-7/0 cells in the presence of a polycyclic aromatic hydrocarbon (PAH), e.g., 3 μM methylcholanthrene, for 5 days [Sreerama and Sladek, 1993b, 1994]), we have demonstrated that ALDH-3 is an important determinant of cellular sensitivity to the oxazaphosphorines [Sreerama and Sladek 1993a,b, 1994]. MCF-7 cells do not contain any of the mixed function oxidases that activate cyclophosphamide, Figure 1. Thus, we used mafosfamide and/or 4-hydroperoxycyclophosphamide rather than cyclophosphamide in all of these and other experiments with cultured MCF-7 cells because these agents, like cyclophosphamide, give rise to 4-hydroxycyclophosphamide, but they do so in the absence of any enzyme involvement, Figure 1.

The above investigations led us to hypothesize that 1) clinical breast cancer cellular resistance to cyclophosphamide and other oxazaphosphorines is the consequence of elevated ALDH-3 levels, 2) ALDH-3 mediates cellular resistance to oxazaphosphorines by catalyzing the oxidative and/or hydrolytic detoxification of these agents, 3) inhibitors of the detoxifying reaction can be identified and utilized to reverse the resistance, 4) hypomethylation of ALDH-3 genomic DNA accounts for oxazaphosphorine- and activated Ah receptor-induced ALDH-3 overexpression, 5) activated Ah receptor-induced ALDH-3 overexpression can only occur in cells that are estrogen receptor-positive, and 6) agents known to induce xenobiotic-metabolizing enzymes via the antioxidant responsive element (ARE) will also induce ALDH-3 overexpression, since ARE is present in the 5'-flanking region of the ALDH-3 gene. Testing of these hypotheses was divided into seven tasks (statement of work), viz., 1) quantify cellular ALDH-3 levels in surgically removed human breast tumor samples, 2) ascertain the ability of ALDH-3s to catalyze the oxidative and/or hydrolytic detoxification of cyclophosphamide (aldophosphamide) at a rate sufficient to account for the oxazaphosphorine-specific acquired resistance exhibited in our model systems, 3) synthesize and identify agents that inhibit the ALDH-3-catalyzed oxidative and/or hydrolytic detoxification of cyclophosphamide (aldophosphamide), 4) evaluate identified inhibitors of the relevant ALDH-3 activity with respect to their ability to sensitize our oxazaphosphorine-resistant models to the oxazaphosphorines, 5) identify the molecular basis for the apparent overexpression of ALDH-3s in our model systems, 6) ascertain the ability of Ah receptor ligands to induce ALDH-3 overexpression and oxazaphosphorine-specific acquired resistance in estrogen receptor-positive and -negative breast cancer cell lines that lack and express Ah receptors, and 7) ascertain the ability of ligands for ARE to

induce ALDH-3 activity and oxazaphosphorine-specific acquired resistance in our model system.

Repository breast tumor samples and culture models, viz., MCF-7/0, MCF-7/OAP and MCF-7/PAH, were chosen to test the hypotheses delineated above. Methods/technology to be used in testing the above-listed hypotheses include immunocytochemistry, ultracentrifugation, column and thin-layer chromatography, HPLC, spectrophotometry to monitor catalytic rates, synthetic organic chemistry, cell culture and colony-forming assays, Northern and Southern blot analysis, methylation-sensitive restriction enzyme diagnosis and receptor binding assays.

We have completed virtually all of the experimental work related to task # 7. Investigations regarding tasks # 1 and 3 are in progress. Investigations relating to tasks # 2, 4, 5 and 6 have yet to be initiated. Results of investigations conducted in months 1 through 12 are summarized below.

BODY

Task # 1: Quantify cellular ALDH-3 levels in surgically removed human breast tumor samples.

Cellular levels of ALDH-3 in nearly 200 surgically removed primary and metastatic human breast tumor samples are being measured by immunocytochemical staining methodology. Correlations between this data and clinical outcomes (in those cases where an oxazaphosphorine was used as part of the therapy) will be sought. ALDH-1 is another known determinant of cellular sensitivity to the oxazaphosphorines [reviewed in Sladek, 1993]. Thus, ALDH-1 levels in breast tumor tissues, together with those of ALDH-3, are more likely to correlate with clinical outcome than are ALDH-3 levels alone. Hence, measurement of ALDH-1 has been added to the original protocol.

Immunocytochemical staining methodology to visualize and semiquantify any ALDH-1 and ALDH-3 present in breast tumor tissue was developed and standardized with the aid of frozen human liver and stomach mucosa samples, and several cultured cell lines, that contained known amounts of ALDH-1 and/or ALDH-3 activities. Specifically, frozen tissue sections were first incubated with polyclonal antibodies specific for ALDH-1 or ALDH-3 and then with a biotin-linked secondary antibody, after which they were incubated with an avidin-biotin complex conjugated to horse radish peroxidase and then with diaminobenzidine and H_2O_2 for color development. This method proved to be highly sensitive and reproducible when used on the frozen breast tumor tissue sections which normally contain significantly lower amounts of aldehyde dehydrogenase as compared to that contained by human liver and stomach mucosa. The optimized procedure is now being used to semiquantify the levels of ALDH-1 and ALDH-3 present in our repository of breast tumor tissue samples.

An approximately 5-fold range of staining intensities has been observed for both ALDH-1 and ALDH-3 in the 15 breast tumor samples tested so far.

Task # 3: Synthesize and identify agents that inhibit the ALDH-3-catalyzed oxidative and/or hydrolytic detoxification of cyclophosphamide (aldophosphamide)

In initial studies, we chose to test whether ethylphenyl(2-formylethyl)phosphinate (EPP) and any of five chlorpropamide analogs would inhibit ALDH-3-catalyzed oxidation of benzaldehyde.

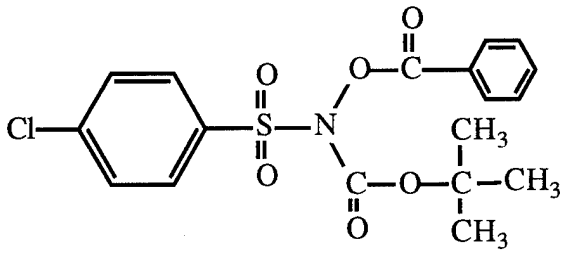
EPP is structurally similar to aldophosphamide. It was synthesized for us by Dr. C.-H. Kwon, St. John's University, Jamaica, NY.

Five chlorpropamide analogs, of which three are carbamate esters (nitroxyl proinhibitors; NPIs), viz., NPI-1, NPI-2 and NPI-3, and two are sulphonylureas (alkylisocyanate proinhibitors; APIs), viz., API-1 and API-2, Figure 3, were synthesized in Dr. H. T. Nagasawa's laboratory [Lee et al., 1992a,b]. The NPIs generate nitroxyl (HNO) as a consequence of ester bond hydrolysis. ALDH-3, like other human aldehyde dehydrogenases, is a bifunctional enzyme, i.e., it catalyzes ester bond hydrolysis as well as the oxidation of aldehydes to acids. Hydrolysis of the NPIs catalyzed by yeast aldehyde dehydrogenase (yALDH) thus giving rise to HNO, and inhibition of yALDH-catalyzed oxidation of acetaldehyde by the newly generated HNO, have been demonstrated [Lee et al., 1992a]. The expectation was that ALDH-3 would also catalyze the hydrolysis of NPIs thereby generating HNO. Preliminary experiments established that HNO inhibits ALDH-3-catalyzed oxidation of benzaldehyde. In contrast, the APIs generate alkylisocyanate without benefit of enzymatic involvement, and this metabolite, too, inhibits yeast, as well as rodent class-2, aldehyde dehydrogenase-catalyzed oxidation of acetaldehyde [Lee et al., 1992a,b].

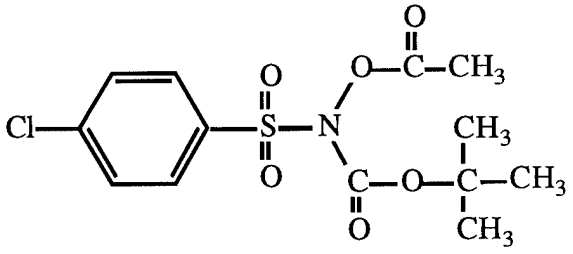
We have generated evidence to support the notion that, although nearly identical, the ALDH-3 present in normal tissues/fluids, e.g., human stomach mucosa and saliva, is different from the ALDH-3 present in breast and other tumor tissues, e.g., colon and salivary gland [reviewed in Sladek et al., 1995; Sreerama and Sladek, 1995a,b,c], i.e., that, putatively, the latter is tumor specific. Hence, the inhibitory potency of the compounds described above was tested against normal cell ALDH-3 as well as against tumor cell ALDH-3. The inhibitory potency of these compounds towards ALDH-1 and ALDH-2 was also determined so that the relative specificity, if any, of these compounds for ALDH-3s, particularly tumor cell ALDH-3, could be assessed. Inhibition of yALDH by these compounds was quantified for comparative purposes since, historically, this enzyme has been used to screen for potential aldehyde dehydrogenase inhibitors. Purified aldehyde dehydrogenases and spectrophotometric assays were used in these investigations.

E. coli [BL21(DE3)pLysS] transfected with pET-19b vector to which human ALDH-1 cDNA was ligated, was provided by Dr. Jan Moreb, University of Florida, Gainsville, FL. Recombinant human ALDH-1 was overexpressed in E. coli by growing them in the presence of isopropylthio-β-D-galactoside (IPTG) and the overexpressed ALDH-1 was purified by Ni-Sepharose CL 6B affinity column chromatography according to the manufacturers protocol [Novagen, Inc., Madison, WI]. A vector, viz., pT7-7, to which human ALDH-2 cDNA was ligated, was provided by Dr. Henry Weiner, Purdue

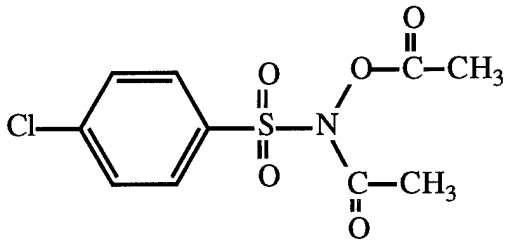
University, Lafayette, IN. The pT7-7 vector was transfected into E. coli [BL21(DE3)] and recombinant human ALDH-2 was overexpressed by growing them in the presence of IPTG. The overexpressed ALDH-2 was purified by ion-exchange chromatography on DEAE-Sephacel followed by affinity chromatography on 5'-AMP-Sepharose CL 6B [Dockham et al., 1992]. Normal cell ALDH-3 and tumor cell ALDH-3 were purified from human stomach mucosa and MCF-7/CAT cells (MCF-7/0 cells cultured in the presence of 30 μM catechol for 5 days; contain >100-fold more ALDH-3 activity as compared to that contained by MCF-7/0 cells cultured in the presence of vehicle alone), respectively, as described previously [Sreerama and Sladek, 1993a; Sreerama et al., 1995a]. yALDH was purchased from Sigma Chemical Company, St. Louis, MO.



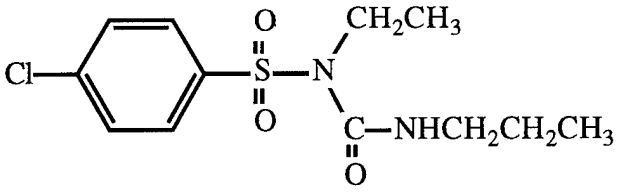
NPI-1



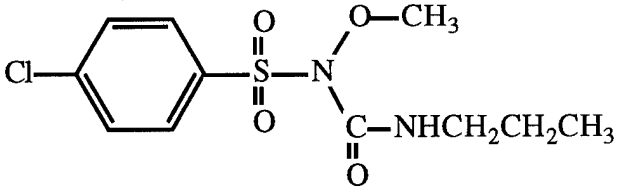
NPI-2



NPI-3



API-1



API-2

NPI-1 = Benzoyloxy[(4-chlorophenyl)sulfonyl]carbamic acid-1,1-dimethylethyl ester

NPI-2 = Acetyloxy[(4-chlorophenyl)sulfonyl]carbamic acid-1,1-dimethylethyl ester

NPI-3 = N-Acetyl N-(acetyloxy)-4-chloro-benzenesulphonamide (designated JAE 34 37/20 in the grant application)

API-1 = 4-Chloro-N-ethyl-N-[(propylamino)carbonyl]benzenesulfonamide

API-2 = 4-Chloro-N-methoxy-N-[(propylamino)carbonyl]benzenesulfonamide

Figure 3. Chlorpropamide analogs (nitroxyl and alkylisocyanate proinhibitors).

EPP did not inhibit any of the human aldehyde dehydrogenases tested; however, it was found to be a relatively good substrate for ALDH-1; Km = 32 μM. It (100 μM) did not potentiate the cytotoxic action of mafosfamide against MCF-7/0 and MCF-7/CAT cells.

NPIs -1 to -3 and APIs -1 and -2 exhibited differential inhibitory potency towards the human aldehyde dehydrogenases, Table 1. The inhibitory constant [Ki] values for NPI-2 inhibition of ALDH-1, ALDH-2, normal cell ALDH-3 and tumor cell ALDH-3 were 7, 370, 30 and 18, μM, respectively. The inhibitory constant [Ki] values for NPI-5 inhibition of ALDH-1, ALDH-2, normal cell ALDH-3 and tumor cell ALDH-3 were 0.4, 0.1, 3.6 and 0.7, μM, respectively.

The differential inhibitory potency of NPIs and APIs towards normal and tumor cell ALDH-3s further supports the notion that tumor cell ALDH-3 is different from the normal cell ALDH-3.

Table 1. Inhibition of aldehyde dehydrogenases by nitroxyl and alkylisothiocyanate proinhibitors*

Test Compound	IC_{50}, μM†				
	ALDH-1	ALDH-2	nALDH-3‡	tALDH-3‡	yALDH‡
NPI-1	25	117	100	75	23
NPI-2	0.75	1300	250	23	18
NPI-3	63	880	710	300	10
API-1	45	6.0	220	45	15
API-2	0.45	0.06	5.0	0.7	0.12

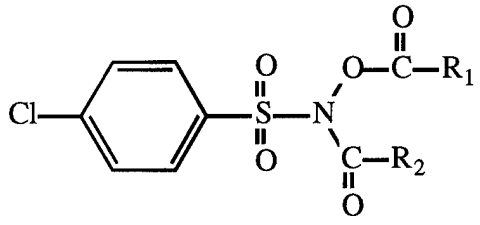
*Purified aldehyde dehydrogenases were preincubated with various concentrations of inhibitors at 37°C for 5 min. Enzyme activity was then measured as described previously [Dockham et al., 1992; Sreerama and Sladek, 1993a, 1994]. Acetaldehyde and NAD, 4 mM each, were substrate and cofactor, respectively, for ALDH-1; acetaldehyde (2 mM) and NAD (4 mM) were substrate and cofactor, respectively, for ALDH-2; benzaldehyde (4 mM) and NAD (1 mM) were substrate and cofactor, respectively, for the two ALDH-3s; and acetaldehyde (0.8 mM) and NAD (4 mM) were substrate and cofactor, respectively, for yALDH.

†Concentration of the test compound required to effect 50% inhibition of catalytic activity.

‡nALDH-3, human normal stomach mucosa ALDH-3 (normal cell ALDH-3); tALDH-3, human breast adenocarcinoma MCF-7/CAT ALDH-3 (tumor cell ALDH-3); and yALDH, yeast aldehyde dehydrogenase.

In addition to the above-described five compounds, two additional series of NPIs (nitroxyl proinhibitors) were synthesized based on prototype compounds **1** and **2** (Figure 4). The starting compound, 4-chloro-N-hydroxybenzenesulfonamide, was synthesized by published methods [Przybylski and Kupryszewski, 1975]. Compound **1a** was made by first acylating with one equivalent of acetyl chloride, which still required a separation procedure to isolate the desired monoacetylated product from the significant bis-acetylated

product, and subsequently reacting the monoacetylated product with benzoyl chloride in the presence of pyridine. Compound **1b** was synthesized directly from 4-chloro-N-hydroxybenzenesulfonamide by reacting it with di-t-butyldicarbonate and triethylamine in tetrahydrofuran. The N-hydroxymethanesulfonamide starting material for compounds in series **2** was synthesized from methanesulfonyl chloride and hydroxylamine according to the method used for 4-chloro-N-hydroxybenzenesulfonamide. This starting material was then acylated with the corresponding alkyl or aryl chloroformate to form compounds **2a-e**. Whether any of these agents inhibit any of the aldehyde dehydrogenases remains to be determined.



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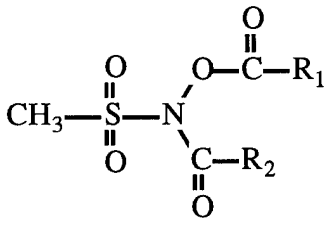

2

1a $R_1 = CH_3$, $R_2 = C_6H_5$
1b $R_1 = R_2 = OC(CH_3)_3$

2a $R_1 = R_2 = OCH_3$
2b $R_1 = R_2 = OCH_2CH_3$
2c $R_1 = R_2 = OCH_2CH_2CH_3$
2d $R_1 = R_2 = OC(CH_3)_3$
2e $R_1 = R_2 = OC_6H_5$

Figure 4. Analogs of NPI-3 (JAE 34 37/20).

Task # 7: Ascertain the ability of ligands for ARE to induce ALDH-3 activity and oxazaphosphorine-specific acquired resistance in our model system.

MCF-7/0 cells were cultured in the presence of agents, viz., phenolic antioxidants (30 μM each for 5 days), known to induce glutathione S-transferase and/or DT-diaphorase via AREs present in the 5'-upstream regions of these enzymes. Cellular levels of ALDH-3 activity were then quantified. Each of the phenolic antioxidants tested induced ALDH-3 activity in MCF-7/0 cells, but the degree of induction varied, Table 2. Immunoblot (specific polyclonal antibodies raised against stomach mucosa ALDH-3 were used) and Northern blot (an oligonucleotide probe specific for the mRNA sequence that codes for the 9 N-terminal end amino acids of stomach mucosa ALDH-3 was used) analyses confirmed the induction of ALDH-3 protein and ALDH-3 mRNA in MCF-7/0 cells treated with catechol (30 μM for 5 days). ALDH-3 induction by catechol was concentration dependent; maximum induction was achieved at a concentration of 60 μM, Figure 5. In addition to MCF-7/0 cells, phenolic antioxidants, e.g., catechol (30 μM for 5 days), induced ALDH-3

in other human breast tumor cells, viz., T-47D, ZR-75-1, MDA-MB-231 and SK-BR-3, as well as in human colon carcinoma cells, viz., HCT 116b and colon C [Sreerama et al., 1995a]. We previously noted that PAHs induced ALDH-3 in breast cancer cells that were estrogen receptor positive, e.g., MCF-7/0, T-47D and ZR-75-1, but not in estrogen receptor negative cells, e.g., MDA-MB-231 and SK-BR-3 [Sreerama and Sladek, 1994]. In contrast, phenolic antioxidants induce ALDH-3 in both estrogen receptor positive and estrogen receptor negative cells [Sreerama et al., 1995a].

Table 2. Class-3 aldehyde dehydrogenase activity in untreated and phenolic antioxidant-treated human breast adenocarcinoma MCF-7/0 cells*

Phenolic Antioxidant	ALDH-3 Activity, mIU/10^7 cells
Control	1.7
Catechol	768
Hydroquinone	438
3,5-Di-tert-butyl-4-hydroxyanisole	163
Ethoxyquin	132
t-Butylhydroquinone	107
2,6-Di-tert-butyl-4-hydroxytoluene	46
Vitamin E	9.0

*Exponentially growing MCF-7/0 cells (1 x 10^5) were cultured in the presence of vehicle (control) or a phenolic antioxidant, 30 μM, for 5 days. They were then harvested and Lubrol-treated whole homogenates (2.5 x 10^4 - 5 x 10^6 cells) were prepared and assayed for aldehyde dehydrogenase activity as described in Sreerama and Sladek [1993a, 1994]. Each value is the mean of four determinations.

PAHs coordinately induced ALDH-3, glutathione S-transferase, DT-diaphorase, UDP-glucuronosyl transferase and cytochrome P450 IA1 in MCF-7/0 cells [reviewed in Sladek et al., 1995], whereas phenolic antioxidants coordinately induced all of the above enzymes except cytochrome P450 IA1, Table 3. Coordinate induction by PAHs is effected via the Ah receptor and xenobiotic responsive elements present in the 5'-upstream region of each of the genes coding for ALDH-3, glutathione S-transferase, DT-diaphorase, UDP-glucuronosyl transferase and cytochrome P450 IA1 [reviewed in Sladek et al., 1995]. Coordinated induction by phenolic antioxidants is effected via an ARE present in the 5'-upstream region of each of the genes coding for ALDH-3, glutathione S-transferase, DT-diaphorase and UDP-glucuronosyl transferase. An ARE is not present in the 5'-upstream region of cytochrome P450 IA1 [reviewed in Sladek et al., 1995].

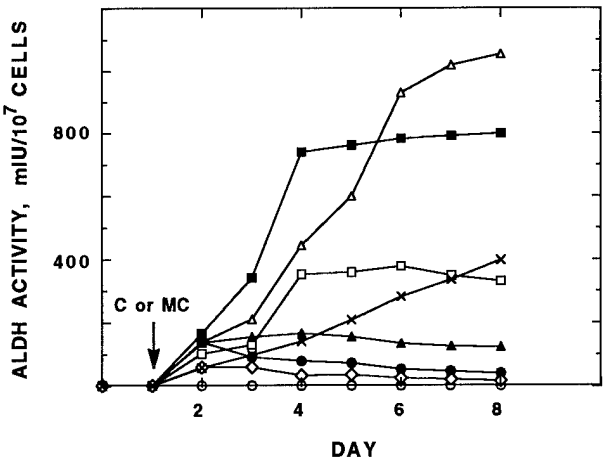

Figure 5. Induction of aldehyde dehydrogenase (ALDH) activity in MCF-7/0 cells by catechol (C): Concentration dependence. Exponentially growing MCF-7/0 cells were continuously exposed to vehicle (○), 3 μM 3-methylcholanthrene (MC) (×), or 3 (●), 10 (□), 30 (■), 60 (△), 100 (▲), or 300 (◇) μM catechol, for 7 days. Lubrol-treated whole homogenates of these cells were then prepared and ALDH activity therein was quantified as described in Sreerama et al [1995a]; benzaldehyde (4 mM) and NADP (4 mM) were used as substrate and cofactor, respectively. Values are means of duplicate determinations made in each of two separate experiments.

The ALDH-3 induced by catechol was purified, Figure 6, and physically and kinetically characterized [Sreerama et al., 1995a]; it was found to be identical to the ALDH-3 present in MCF-7/PAH cells [Sreerama and Sladek, 1994].

MCF-7/0 cells cultured in the presence of catechol (30 μM for 5 days) were less sensitive to mafosfamide than were untreated MCF-7/0 cells, Figure 7. Sensitivity to phosphoramide mustard was not decreased [Sreerama et al., 1995a]. The relative insensitivity to mafosfamide exhibited by these cells was transient since, following the removal of catechol from the culture medium, sensitivity to mafosfamide, as well as ALDH-3 activity and the cellular content of ALDH-3 mRNA, returned to basal levels within 10 days, Figure 7.

The ALDH-3 isolated from MCF-7/CAT cells, like the ALDH-3 isolated from other tumor sources [reviewed in Sladek et al., 1995], was found to be a subtle, putatively tumor specific, variant of the ALDH-3 isolated from normal cells/secretions, e.g., stomach mucosa, parotid gland and whole saliva [reviewed in Sladek et al., 1995]. Tumor cell ALDH-3, relative to normal cell ALDH-3, was more able to catalyze the oxidation of aldophosphamide [reviewed in Sladek et al., 1995] and, thus, the detoxification of oxazaphosphorines [Sreerama and Sladek, 1995]. It was also more sensitive to inhibition by chlorpropamide analogs, Table 1. Physical and all other catalytic properties appeared to be identical.

Table 3. Class-3 aldehyde dehydrogenase, DT-diaphorase, glutathione S-transferase, UDP-glucuronosyl transferase and cytochrome P450 IA1 activities in untreated and catechol-treated human breast adenocarcinoma MCF-7/0 cells*

Enzyme	mIU/10^7 cells		Treated/Control
	Control	Treated	
NAD-ALDH-3	1.5 ± 0.1	381 ± 13	254
NADP-ALDH-3	1.7 ± 0.1	768 ± 43	452
DT-diaphorase	77 ± 4	6395 ± 748	83
Glutathione S-transferase	24 ± 2	250 ± 11	10
UDP-glucuronosyl transferase	0.04 ± 0.004	0.07 ± 0.004	2
Cytochrome P450 IA1	0.032 ± 0.003	0.034 ± 0.002	1

*Exponentially growing MCF-7/0 cells (1 x 10^5) were cultured in the presence of vehicle (control) or 30 μM catechol (treated) for 5 days. They were then harvested and Lubrol-treated whole homogenates (2.5 x 10^4 - 5 x 10^6 cells) were prepared and assayed for ALDH-3, DT-diaphorase, and glutathione S-transferase activities as described in Sreerama and Sladek [1993a, 1994]. In addition, microsomal fractions (2.5 x 10^6 - 1 x 10^7) were prepared and assayed for UDP-glucuronosyl transferase and cytochrome P450 IA1 activities as described in Mackenzie and Hanninen [1980] and Sreerama et al [1995a], respectively. Each value is the mean ± standard error of four determinations.

As in MCF-7/CAT cells, coordinately elevated levels of ALDH-3, glutathione S-transferases, DT-diaphorase and UDP-glucuronosyl transferase, but not of cytochrome P450 IA1, were also found in MCF-7/OAP cells. As a consequence of increased enzyme levels (relative to those in MCF-7/0 cells), the sensitivity of MCF-7/PAH, MCF-7/CAT and MCF-7/OAP cells to certain anticancer drugs was (relative to the sensitivity of MCF-7/0 cells to these same drugs) markedly altered. Thus, the rate at which some drugs were bio**inactivated**, e.g., mafosfamide, melphalan and mitoxantrone, was increased and sensitivity to these drugs was decreased (multidrug resistance), and the rate at which some other drugs were bio**activated**, e.g., ellipticine and the indoloquinone EO9, was also increased but sensitivity to these drugs was increased (collateral sensitivity) [Rekha and Sladek, 1996]. Xenobiotic-induced multienzyme-mediated multidrug resistance/collateral sensitivity thus demonstrated is mechanistically different from, and pertains to a largely different group of anticancer agents than is/does, the multidrug resistance effected by multidrug resistance-associated protein (MRP) or the P-glycoprotein coded for by the *mdr* 1 gene. Indeed, multidrug resistance mediated by enzymes simultaneously induced by a xenobiotic is a newly recognized mechanism by which multidrug resistance can be effected.

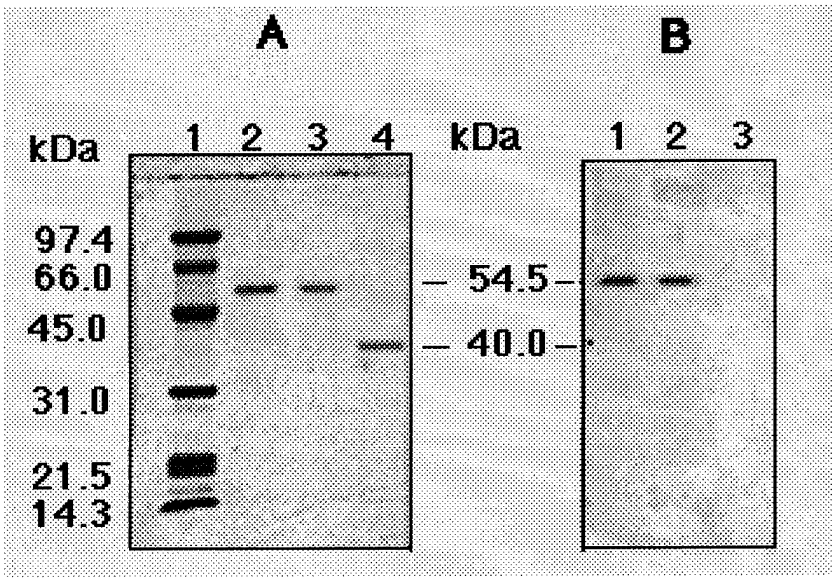

Figure 6. Class-3 aldehyde dehydrogenase purified from catechol-treated MCF-7/0 cells: subunit molecular weight and recognition of the denatured enzyme by anti-stomach mucosa ALDH-3 IgY. Induction of ALDH-3 by catechol, and the subsequent purification of this enzyme, were as described in Sreerama et al [1995a] and in Tables 2 & 3. Panel **A**: SDS-PAGE of molecular weight markers (*Lane 1*) and 5 µg each of purified stomach mucosa ALDH-3 (*Lane 2*), catechol-induced enzyme (*Lane 3*), and MCF-7/OAP ALDH-3 (*Lane 4*) was as described in Sreerama and Sladek [1993a]. Molecular weight markers were lysozyme (14.3 kDa), trypsin inhibitor (21.5 kDa), carbonic anhydrase (31 kDa), ovalbumin (45 kDa), BSA monomer (66 kDa) and phosphorylase B (97.4 kDa). Proteins in each lane were visualized by staining with Coomassie Brilliant Blue R-250. A plot of log M_r *versus* mobility was used to estimate the subunit molecular weight of the catechol-induced enzyme [Sreerama and Sladek, 1993a]. Panel **B**: Purified stomach mucosa ALDH-3 (*Lane 1*), purified catechol-induced enzyme (*Lane 2*), and purified MCF-7/OAP ALDH-3 (*Lane 3*) were submitted to SDS-PAGE and electrotransferred onto a Immobilon-PVDF transfer membrane; attempted visualization of the denatured enzymes with anti-stomach mucosa ALDH-3 IgY was as described in Sreerama and Sladek [1993a]. Placed on the gel were 5 µg of each purified enzyme.

The agents that coordinately induce the above enzymes are abundantly present in human diet, e.g., beverages such as coffee and vegetables such as broccoli [summarized in Sreerama et al., 1995b]. We have demonstrated that consumption of large amounts of coffee or broccoli by human subjects results in the coordinated elevation of ALDH-3, glutathione S-transferases and DT-diaphorase levels in saliva [Sreerama et al., 1995b]. Thus, assuming that the xenobiotic-induced coordinated elevation of salivary enzymes mirrors the coordinated elevation of these enzymes in various cancer cells, dietary and other environmental factors could greatly influence the therapeutic efficacy of anticancer drugs that are bioinactivated or bioactivated by these enzymes [Sreerama et al., 1995b; Rekha and Sladek 1996].

In light of the foregoing, we initiated an investigation in which glutathione S-transferase and DT-diaphorase, as well as ALDH-1 and ALDH-3, levels in normal and malignant breast tissue samples (nearly 130) procured from the Cooperative Human Tissue Network, Midwestern Division, Columbus, Ohio, are to be quantified. Catalytic assays (spectrophotometric) and enzyme-linked immunosorbent assays (ELISAs) are to be used

for this purpose. Catalytic assays (spectrophotometric) quantifying ALDH-1, ALDH-3, glutathione S-transferase and DT-diaphorase levels were as described previously [Sreerama and Sladek, 1993a, 1994].

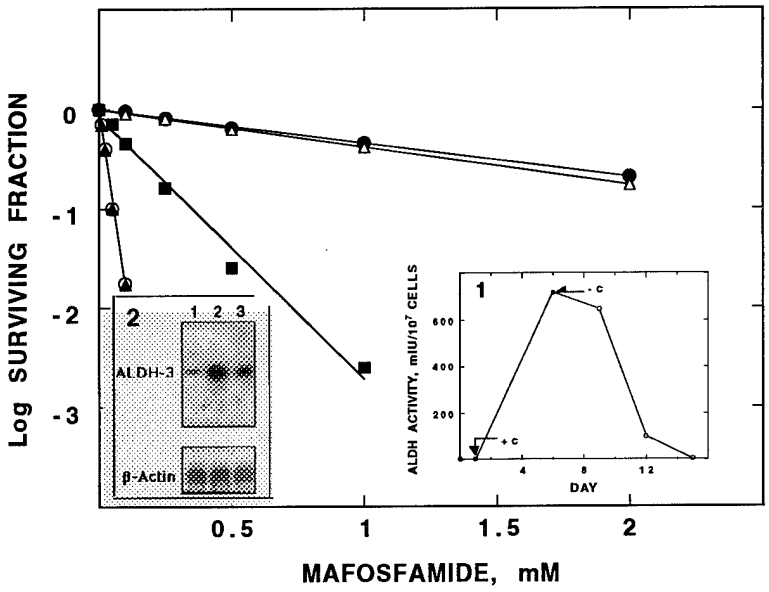

Figure 7. Effect of adding and then removing catechol from the culture medium on the sensitivity of MCF-7/0 cells to mafosfamide. Exponentially growing MCF-7/0 cells were cultured in the presence of 30 μM catechol (C) for 5 days. At the end of this time, cells were harvested, washed, resuspended in catechol-free growth medium, and cultured for an additional 9 days. Sensitivity to mafosfamide was determined as described in Sreerama and Sladek [1993a, 1994] on days 0 (○), 6(●), 9 (△), 12 (■), and 15 (▲). Each point is the mean of measurements on triplicate cultures. LC_{90} values (concentrations of drug required to kill 90% of cells) obtained from these plots were 55 (○), >2000 (●), >2000 (△), 370 (■), and 55 (▲) μM. *Inset 1*: Aldehyde dehydrogenase activity at corresponding time points. NADP (4 mM)-linked aldehyde dehydrogenase-catalyzed oxidation of benzaldehyde (4 mM) was quantified in Lubrol-treated whole homogenates, as described in Sreerama et al [1995a], at the times indicated. Reaction mixtures (1 ml) contained whole homogenates prepared from 1.5 x 10^5 to 1 x 10^7 cells. Each value is the mean of duplicate determinations. *Inset 2*: Northern blot analysis of poly(A)$^+$-enriched RNA isolated from MCF-7/0 cells (*Lane 1*), MCF-7/0 cells treated with catechol (30 μM for 5 days) (*Lane 2*), and MCF-7/0 cells treated with catechol (30 μM for 5 days) and then cultured in the absence of catechol for 3 days (*Lane 3*). Isolation of total RNA, poly(A)$^+$-enriched RNA, electrophoresis of poly(A)$^+$-enriched RNA, transfer of poly(A)$^+$-enriched RNA onto a Zeta-Probe nylon membrane, and probing of the blotted membrane with ^{32}P-labeled oligonucleotide specific for human stomach mucosa ALDH-3 and with full-length β-actin cDNA were described in Sreerama et al [1995a]. Placed on the gels were 10 μg of poly(A)$^+$-enriched RNA in each case.

To date, quantification of ALDH-1, ALDH-3, glutathione S-transferase (global) and DT-diaphorase levels in 15 normal breast, and 80 primary and metastatic breast tumor, tissue samples has been completed, Table 4 and Sladek and Sreerama [1995]. Glutathione S-transferase and DT-diaphorase levels varied widely, and, as in the investigations reported under task # 1, ALDH-1 and ALDH-3, each also varied widely. However, coordinated elevation of ALDH-3, glutathione S-transferase (global) and DT-diaphorase levels was only occasionally observed.

Table 4. ALDH-1, ALDH-3, DT-diaphorase and glutathione S-transferase activities in human normal breast, and primary and metastatic breast tumor, tissue samples*

Enzyme	mIU/g Breast Tissue			
	Normal		Malignant	
	Mean ± SD	Range	Mean ± SD	Range
ALDH-1	17 ± 14	2 - 49	35 ± 35	3 - 188
ALDH-3	10 ± 5	2 - 19	23 ± 40	1 - 247
DT-Diaphorase	522 ± 623	23 - 2450	1410 ± 1670	10 - 7900
Glutathione S-Transferase	938 ± 885	217 - 3600	2380 ± 1900	137 - 8480

*Preparation of soluble (105,000 x g supernatant) fractions from human normal breast tissues (n = 15) and breast tumor tissues (n = 80), and quantification of ALDH-1, ALDH-3, DT-diaphorase, and glutathione S-transferase activities were as described in Sreerama and Sladek [1993a, 1994]. Acetaldehyde and NAD, 4 mM each, were the substrate and cofactor, respectively, for ALDH-1. Benzaldehyde and NADP, 4 mM each were the substrate and cofactor, respectively, for ALDH-3. 1-Chloro-2,4-dinitrobenzene and glutathione, 1 mM each, were the substrate and cofactor respectively, for glutathione S-transferase. The substrate, cofactor and inhibitor for DT-diaphorase were, respectively, 2,6-dichlorophenol-indophenol (40 μM), NAD(P)H (160 μM) and dicumarol (10 μM).

At the time that the grant proposal was submitted, we expected to address task # 7 during months 36-48 (Statement of Work). The potential significance of identifying a new mechanism by which ALDH-3 can be induced, viz., by phenolic antioxidants via an ARE, and the therapeutic consequences thereof, persuaded us to test this hypothesis ahead of schedule.

Parts of the investigations described herein were conducted during the time period between submission of this grant proposal and its initiation on October 1, 1994; funding during that interval was by grants awarded by the National Institutes of Health and Bristol-Myers Squibb Co.

CONCLUSIONS

ALDH-1 and ALDH-3 catalyze the detoxification of oxazaphosphorines. Elevated levels of either of these enzymes in cultured tumor cell models account for the decrease in cellular sensitivity to oxazaphosphorines. Elevated levels of these enzymes in tumor tissue are expected to result in clinical resistance to oxazaphosphorines. The broad range of ALDH-1 and ALDH-3 levels found in the breast tumor samples tested thus far suggests that these enzymes are operative determinants of cellular sensitivity to cyclophosphamide and other oxazaphosphorines in the clinic.

Human tumor cell ALDH-3 was found to be more sensitive to inhibition by each of five chlorpropamide analogs than was human normal cell ALDH-3. This further supports the notion that tumor cell ALDH-3 is a subtle, putatively tumor-specific, variant of normal cell ALDH-3. Thus, it may be possible to develop a clinically useful selective inhibitor of tumor cell ALDH-3 thereby allowing the selective sensitization of tumor cells to cyclophosphamide and other oxazaphosphorines. A tumor-specific ALDH-3 would also have diagnostic potential.

Phenolic antioxidants were found to rapidly, coordinately and reversibly induce ALDH-3, glutathione S-transferase, DT-diaphorase and UDP-glucuronosyl transferase in cultured breast and other tumor cells, the consequence of which was that such cells became less sensitive to certain anticancer drugs (multidrug resistance) and more sensitive to others (collateral sensitivity). These findings should be viewed as greatly expanding the number of recognized dietary, environmental and pharmaceutical agents that can potentially influence the sensitivity of breast and other tumor cells to cyclophosphamide, other oxazaphosphorines and still other antitumor agents since, in addition to phenolic antioxidants which themselves are abundantly present in a number of dietary materials, a number of other dietary, etc., agents are thought to effect enzyme induction via the signaling mechanism used by the phenolic antioxidants. Assuming that what we observed in our cell culture models reflects what happens clinically, the relevance of the foregoing with regard to the rational selection of cancer chemotherapeutic agents is obvious.

A broad range of glutathione S-transferase and DT-diaphorase, as well as of ALDH-1 and ALDH-3, levels was found in human breast tumor tissue samples. Supporting the notion of coordinated induction of ALDH-3, glutathione S-transferase and DT-diaphorase by dietary, etc., agents in human tumor tissue was the fact that, in some cases, the levels of all three were elevated. Further supporting this notion is our observation that salivary levels of all three enzymes are elevated in individuals consuming coffee or broccoli, each of which is known to contain agents that coordinately induce each of these enzymes.

Moreover, assuming that salivary levels of these enzymes reflect their tissue levels, quantification of the salivary content of one of them, e.g., ALDH-3, a noninvasive undertaking, could be useful in deciding whether to use cyclophosphamide or another drug that is detoxified by one of these enzymes, as opposed to a drug, e.g., mitomycin C, that is bioactivated by one of them.

In addition to catalyzing the detoxification/activation of antineoplastic drugs, ALDH-3, glutathione S-transferases and DT-diaphorase are known to catalyze the detoxification of various carcinogens. Therefore, quantification of one or more of these enzymes in saliva could be useful in a) preliminarily assessing the chemo**preventive** potential of various diets and drugs and b) identifying the optimum dose and schedule to be used for any putatively chemopreventive diets or drugs of interest, i.e., those suspected of being chemopreventive by the virtue of their ability to induce these enzymes. Quantification of salivary levels of these enzymes may also be useful as an indicator when exposure to carcinogenic/teratogenic/otherwise toxic environmental/industrial/dietary agents that induce these enzymes is suspected.

The changes incorporated into the original protocols to better address hypotheses 1 have been discussed in sections titled Task # 1 and Task # 7. Task # 2 (Statement of Work), originally planned to be tested during months' 1-24, will be addressed during months' 12-36.

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*Included in the appendix (reprints enclosed).

APPENDIX
(Reprints Enclosed)

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IDENTIFICATION OF HUMAN LIVER ALDEHYDE DEHYDROGENASES THAT CATALYZE THE OXIDATION OF ALDOPHOSPHAMIDE AND RETINALDEHYDE*

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Abstract—Biotransformation of the biologically and pharmacologically important aldehydes, retinaldehyde and aldophosphamide, is mediated, in part, by NAD(P)-dependent aldehyde dehydrogenases that catalyze the oxidation of the aldehydes to their respective acids, retinoic acid and carboxyphosphamide. Not known at the onset of this investigation was which of the several known human aldehyde dehydrogenases (ALDHs) catalyze these reactions. Thus, human liver aldehyde dehydrogenases were chromatographically resolved and the ability of each to catalyze the oxidation of retinaldehyde and aldophosphamide was assessed. Only one, namely ALDH-1, catalyzed the oxidation of retinaldehyde; the K_m value was 0.3 μM. Three, namely ALDH-1, ALDH-2 and succinic semialdehyde dehydrogenase, catalyzed the oxidation of aldophosphamide; K_m values were 52, 1193, and 560 μM, respectively. ALDH-4, ALDH-5 and betaine aldehyde dehydrogenase did not catalyze the oxidation of either aldophosphamide or retinaldehyde. ALDH-1 and succinic semialdehyde dehydrogenase accounted for 64 and 30%, respectively, of the total hepatic aldehyde dehydrogenase-catalyzed aldophosphamide (160 μM) oxidation. ALDH-1-catalyzed oxidation of aldophosphamide was noncompetitively inhibited by chloral hydrate; the K_i value was 13 μM. ALDH-2- and succinic semialdehyde dehydrogenase-catalyzed oxidation of aldophosphamide was relatively insensitive to inhibition by chloral hydrate. These observations strongly suggest an important *in vivo* role for ALDH-1 in the catalysis of retinaldehyde and aldophosphamide biotransformation. Succinic semialdehyde dehydrogenase-catalyzed biotransformation of aldophosphamide may also be of some *in vivo* importance.

NAD(P)-linked aldehyde dehydrogenases (AHD; ALDH‡) catalyze the biotransformation of a wide variety of biologically and pharmacologically important aldehydes [1, 2]. For example, acetaldehyde, a toxic intermediate generated during ethanol metabolism, and aldophosphamide, the penultimate aldehyde metabolite of the anticancer drug cyclophosphamide, are detoxified by aldehyde dehydrogenase-catalyzed oxidation to the corresponding carboxylic acids [2–4]. Further, aldehyde dehydrogenases catalyze the oxidation (bioactivation) of retinaldehyde, a vitamin A metabolite and cleavage product of β-carotene, to retinoic acid, a potent modulator of cell growth and differentiation [5–8].

Mouse liver contains eleven aldehyde dehydrogenases (AHDs 1–3, 5, 7–13), plus an additional NAD-dependent enzyme, xanthine oxidase (dehydrogenase form), that catalyze the NAD(P)-dependent oxidation of aldophosphamide, acetaldehyde, benzaldehyde, and/or octanal to their respective carboxylic acids [9]. At least two additional aldehyde dehydrogenases (AHDs 4 and 6) are not found in liver but are present in other mouse tissues [10]. AHD-4 and nine of the hepatic enzymes catalyze the oxidation of aldophosphamide to carboxyphosphamide [9]. Xanthine oxidase (dehydrogenase form) and two hepatic aldehyde dehydrogenases catalyze the oxidation of retinaldehyde to retinoic acid [8]. AHD-2, the major cytosolic aldehyde dehydrogenase, catalyzes the bulk of the total hepatic oxidation of both aldophosphamide and retinaldehyde. Apparent homologs of some of the mouse enzymes have been found in human livers (Table 1).

Five aldehyde dehydrogenases, viz. ALDHs 1–5, that catalyze the oxidation of acetaldehyde and benzaldehyde to their corresponding acids have been identified in human livers [2, 4, 12]. Also present in this tissue are at least two relatively substrate-specific "aldehyde dehydrogenases", viz. betaine aldehyde dehydrogenase (BADH) [13] and γ-aminobutyraldehyde dehydrogenase [14]. Finally, yet another

* Descriptions of parts of this investigation have appeared in abstract form [Dockham *et al.*, *Pharmacologist* **32**: 156, 1990; Lee *et al.*, *Pharmacologist* **32**: 156, 1990] and in the proceedings of a workshop [Sladek *et al.*, In: *Advances in Experimental Medicine and Biology* (Eds. Weiner H, Wermuth B and Crabb DW), Vol. 284, pp. 97–104. Plenum Press, New York, 1990].

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‡ Abbreviations: AHD, mouse aldehyde dehydrogenase; ALDH, human aldehyde dehydrogenase; MES, 2-[*N*-morpholino]-ethanesulfonic acid; pI, isoelectric point; SDS, sodium dodecyl sulfate; BADH, betaine aldehyde dehydrogenase; and SSDH, succinic semialdehyde dehydrogenase.

Table 1. Human homologs of mouse aldehyde dehydrogenases*

Mouse	Human†	Human‡	Enzyme type§
AHD-1	ALDH-4	ALDH-IV	Glutamic-γ-semialdehyde dehydrogenase
AHD-2	ALDH-1	ALDH-II	Low K_m cytosolic
AHD-3	ALDH-5	ALDH-V	Microsomal
AHD-4	ALDH-3‖	ALDH-III‖	High K_m cytosolic
AHD-5	ALDH-2	ALDH-I	Low K_m mitochondrial
AHD-9	BADH	BADH	Betaine aldehyde dehydrogenase
AHD-12	SSDH	SSDH	Succinic semialdehyde dehydrogenase

* According to Manthey *et al.* [9] and references cited therein.
† Nomenclature used in this manuscript.
‡ Nomenclature used by Manthey *et al.* [9].
§ K_m refers to the K_m obtained when acetaldehyde is the substrate.
‖ ALDH-3 may be the human homolog of AHD-4 [11] but this remains to be established.

relatively substrate-specific "aldehyde dehydrogenase", viz. succinic semialdehyde dehydrogenase (SSDH), while heretofore not reported in human liver, has been identified in human brain [15] and in mouse liver [9].

Not known is which of the human aldehyde dehydrogenases catalyze the oxidation of aldophosphamide and retinaldehyde to their corresponding acids. The present investigation was designed to identify these enzymes and to determine the relative contribution of each to the oxidation of aldophosphamide and retinaldehyde in human liver samples obtained from transplant donors.

MATERIALS AND METHODS

Materials

4-Hydroperoxycyclophosphamide was provided by Dr. Jorge Pöhl, Asta-Werke AG, Bielefeld, FRG. Acetaldehyde, benzaldehyde, octanal, all-*trans*-retinaldehyde (95%), all-*trans*-retinoic acid, tetraphenylethylene, dimethyl sulfoxide (99+%), and methyl sulfide (99+%) were purchased from the Aldrich Chemical Co., Milwaukee, WI. As judged by HPLC/spectrophotometry [8], all-*trans*-retinaldehyde was retinoic acid-free but *cis*-retinaldehyde (<1% of total) was sometimes present. NAD^+, $NADP^+$, NADH, glutathione (reduced form), pyrazole, pyridoxal HCl, betaine aldehyde, succinic semialdehyde, γ-aminobutyraldehyde diethylacetal (90%), DL-Δ^1-pyrroline-5-carboxylate-2,4-dinitrophenylhydrazone, xanthine, bovine serum albumin (crystallized and lyophilized), Lubrol®, Reactive Blue 2-Sepharose CL-6B, and silica gel (70–220 mesh) were purchased from the Sigma Chemical Co., St. Louis, MO. YM-30 ultrafiltration membranes were purchased from Amicon Division, W. R. Grace & Co., Danvers, MA. DEAE-Sephacel, 5′-AMP-Sepharose, Polybuffer Exchanger 94, Polybuffer 74, PD-10 columns, Ampholine PAGplates® (pH 3.5 to 9.5) and an isoelectric point marker kit were purchased from Pharmacia LKB Biotechnology, Piscataway, NJ. EDTA and HPLC-grade solvents were obtained from Fisher Scientific, Ltd., Los Angeles, CA, and the latter were filtered through 0.45 μm Durapore filters (Millipore, Bedford, MA) prior to use. The Bio-Rad Protein Assay Dye Reagent Concentrate was purchased from Bio-Rad Laboratories, Richmond, CA. Chloral hydrate was obtained from the University of Minnesota Hospital Pharmacy, Minneapolis, MN.

Aldophosphamide was generated in aqueous solution by the chemical reduction of 4-hydroperoxycyclophosphamide as described previously [9] except that purer (99+% vs 98%) methyl sulfide was used as the reducing agent. Glutamic-γ-semialdehyde was prepared from DL-Δ^1-pyrroline-5-carboxylate-2,4-dinitrophenylhydrazone as described by Mezl and Knox [16]. γ-Aminobutyraldehyde was prepared fresh daily from γ-aminobutyraldehyde diethylacetal and was partially purified by passage through a 1.0 × 6.5 cm silica gel column according to the procedure of Ambroziak and Pietruszko [17]. It was held at pH 6.5 until just before use.

Human liver samples were obtained from three Caucasian donors through the Liver Tissue Procurement and Distribution System, University of Minnesota, Minneapolis, MN. Donors A and B died as a result of injuries sustained in motor vehicle accidents. An intracerebral aneurysm lead to the death of Donor C. Age and sex of each donor are given in Table 2. All of the donors had been maintained by a life-support system until organ removal. The supplier certified all livers as nonpathological. Liver samples obtained from Donors A and B were kept at 0–4° and were delivered to us within 12 hr of donation. The former, and subcellular fractions thereof, were assayed immediately for aldehyde dehydrogenase activity, that is, without ever being frozen. A portion of the latter was homogenized immediately and submitted to centrifugation to obtain 105,000 *g* soluble and particulate fractions which were then assayed immediately for aldehyde dehydrogenase activity, that is, without ever being frozen; the remaining portion was frozen at −70° and then stored at this temperature until used. The liver sample obtained from Donor C was frozen at −70° within 4 hr of donation and was stored at this temperature until used.

Preparation of hepatic fractions

Human liver 105,000 *g* soluble, and solubilized

particulate, fractions were prepared as previously described for mouse liver [9] except that 0.3% Lubrol®, rather than 0.3% deoxycholate, was used to solubilize 105,000 *g* particulate fractions. Lubrol®-treated whole homogenates were prepared as follows. Liver was cut into 2-mm slices and homogenized in ice-cold homogenization medium (1.15% KCl solution containing 0.3% Lubrol® and 1.0 mM EDTA, pH 7.4) with a Dounce homogenizer. Homogenates (20%) were centrifuged at 105,000 *g* and 4° for 60 min. The supernatants were assayed for protein content and aldehyde dehydrogenase activity, and were submitted to anion exchange chromatography or were frozen at −70° and thawed immediately prior to chromatography or electrophoresis.

Chromatographic resolution and semipurification of human hepatic aldehyde dehydrogenases

DEAE-Sephacel, 5′-AMP-Sepharose, and chromatofocusing column chromatography were performed at 4° as described previously [9]. Dye resin affinity column chromatography with Reactive Blue 2-Sepharose CL-6B was performed at 4° utilizing a 20 cm/hr linear flow rate and a 1.5 × 8 cm column. Protein concentrations of samples loaded onto columns never exceeded 20 mg/mL and typically were much less. Elution of aldehyde dehydrogenase activity off these columns was monitored using the spectrophotometric aldehyde dehydrogenase assay described previously [9]. The NaCl concentration of selected eluate fractions was determined with an Atago refractometer. The pH of selected eluate fractions was determined with a Corning glass pH electrode. Where indicated, the volume of pooled eluate fractions was reduced using an Amicon ultrafiltration stirred-cell apparatus fitted with a YM-30 membrane and pressurized under nitrogen. Transfer of enzyme fractions from one salt/buffer solution to another was accomplished with Pharmacia PD-10 gel filtration columns. Additional details are presented in the figure legends and in Results.

Protein determination

Elution of protein from chromatography columns was routinely monitored at 280 nm with an ISCO UA-5 absorbance monitor. The protein content of crude tissue fractions and selected column pools was determined by the Coomassie Brilliant Blue dye binding assay [18] using commercially available Bio-Rad Protein Assay Reagent and bovine serum albumin as the standard.

Isoelectric focusing

Isoelectric focusing was carried out as described previously [9] except that commercially available Ampholine PAGplates® (pH 3.5 to 9.5) were used. Substrates used to visualize aldehyde dehydrogenases were acetaldehyde (4 mM), glutamic-γ-semialdehyde (200 μM), octanal (100 μM), betaine aldehyde (50 μM), and succinic semialdehyde (100 μM).

Assays for aldehyde dehydrogenase activity

Except that a Beckman DU-70 recording spectrophotometer was used to monitor the appearance of NAD(P)H at 340 nm, spectrophotometric and HPLC/spectrophotometric assays were used as described previously [8, 9] to quantify aldehyde dehydrogenase activity at 37°. The reaction mixture (1 mL, pH 8.2) contained 4 mM NAD, 32 mM tetrasodium pyrophosphate, 0.1 mM pyrazole, 5 mM glutathione, 1 mM EDTA, the substrate of interest, and crude fraction or (semi)purified aldehyde dehydrogenase. Rates expressed per g liver are per g wet weight liver. The freezing, storage and thawing conditions were such that the wet weight of liver that had been frozen did not differ from that obtained before freezing.

Kinetic analysis

Prior to kinetic analysis (as well as to isoelectric focusing), (semi)purified enzymes were first separated (when necessary) from polybuffer and then were transferred, with the aid of PD-10 columns, into 20 mM triethanolamine buffer, pH 7.4, containing 1.0 mM dithiothreitol, and 0.1 mM EDTA; subsequently, these preparations were adjusted to 25% glycerol. Typically, 100 μL of these preparations was used in the aldehyde dehydrogenase assay reaction mixture. K_m, and where indicated, V_{max}, values for the catalysis of retinaldehyde (5 μM), acetaldehyde (10 μM), octanal (10 μM), betaine aldehyde (10 μM), and succinic semialdehyde (10 μM) oxidation by ALDH-1, ALDH-2, ALDH-5, BADH, and SSDH, respectively, were determined by the integrated Michaelis method of analysis of single enzyme progress curves [19]. Double-reciprocal plots of initial rates versus substrate concentrations were used to estimate all other K_m and V_{max} values. Four to ten substrate concentrations were used to generate each pair of K_m and V_{max} values. Wilkinson weighted linear regression analysis [20] was used to fit lines to the double-reciprocal plot values. Computer-assisted unweighted regression analysis was carried out using the STATView® (Brainpower, Inc., Calabas, CA) statistical program to generate all other straight line functions.

RESULTS

Aldehyde dehydrogenase activity was measured in Lubrol®-solubilized whole homogenates prepared from liver samples obtained from each of three donors (Table 2). The mean aldehyde dehydrogenase activities were 7.1, 2.1 and 0.3 μmol/min/g liver when acetaldehyde (4 mM), aldophosphamide (160 μM) and retinaldehyde (25 μM), respectively, were the substrates. A fourth sample, obtained from yet another donor and held at 0–4° for more than 36 hr after surgical removal, showed approximately 30% of the enzyme activities observed in Donors A, B and C (data not presented). As judged by isoelectric focusing patterns, all donors exhibited the normal Caucasian phenotype [2] with regard to NAD-dependent acetaldehyde-metabolizing enzymes (data not shown). Approximately 80% of the total enzyme-catalyzed oxidation of retinaldehyde to retinoic acid in each sample was NAD dependent (data not shown). Pyridoxal (1 mM), a known inhibitor of aldehyde oxidase, inhibited 44% of

Table 2. Aldehyde dehydrogenase activity in Lubrol®-solubilized whole homogenates obtained from human livers*

Donor	Age (years)	Sex	Aldehyde dehydrogenase activity (μmol/min/g liver)		
			Acetaldehyde	Aldophosphamide	Retinaldehyde
A	2.5	Male	6.4	2.4	0.3
B	22	Male	7.5	1.8	0.3
C	51	Female	7.4	2.2	0.3

* Donor tissue was held at 0–4° for less than 12 hr and assayed for enzyme activity (Donor A), or was stored at −70° for several weeks (Donors B and C), prior to assay. Livers were homogenized in the presence of 0.3% Lubrol® and the 105,000 *g* supernatant fractions prepared from these homogenates were assayed for NAD-dependent aldehyde dehydrogenase activity as described in Materials and Methods. The spectrophotometric assay was used to determine enzyme activity when acetaldehyde (4 mM) and aldophosphamide (160 μM) were the substrates. The HPLC/spectrophotometric assay was used to determine enzyme activity when retinaldehyde (25 μM) was the substrate. Values are means of two determinations.

Table 3. Aldehyde dehydrogenase activity in soluble, and solubilized particulate, fractions obtained from human livers*

Substrate	Aldehyde dehydrogenase activity (μmol min/g liver)					
	Donor A		Donor B		Donor C	
	Soluble	Particulate	Soluble	Particulate	Soluble	Particulate
Acetaldehyde	3.7	2.6	4.6	4.1	6.1	1.1
Aldophosphamide	2.6	0.8	2.7	0.5	2.5	0.2
Retinaldehyde	0.4	0.0	0.5	0.0	ND†	ND

* Soluble, and solubilized particulate, fractions were prepared from donor livers that had been held at 0–4° for less than 12 hr (Donors A and B), or that had been stored at −70° for several weeks (Donor C), prior to cell fractionation and assay. The spectrophotometric assay was used to determine enzyme activity when acetaldehyde (4 mM) and aldophosphamide (160 μM) were the substrates. The HPLC/spectrophotometric assay was used to determine enzyme activity when retinaldehyde (25 μM) was the substrate. Values are from single, or means of duplicate, determinations.

† ND: not determined.

the retinaldehyde oxidation catalyzed by NAD-independent enzymes present in a 105,000 *g* soluble fraction prepared from Donor B liver that had never been frozen.

Aldehyde dehydrogenase activity was also measured in 105,000 *g* soluble, and solubilized particulate, fractions prepared from liver samples that had never been frozen (Donors A and B) and a sample that had been frozen (Donor C) (Table 3). In agreement with the experience of others [21], freezing apparently affected the distribution of aldehyde dehydrogenase activity between the soluble and solubilized particulate fractions. Thus, for example, about 45% of total aldehyde dehydrogenase-catalyzed acetaldehyde oxidation occurred in the particulate fraction when the liver sample had never been frozen (Donors A and B), but only about 15% did so when it had (Donor C). Isoelectric focusing analysis showed an increased presence of particulate enzymes in the soluble fraction when previously frozen liver was the source of these fractions as compared to that observed when liver samples that had never been frozen were the source (data not shown). It should also be noted that enzyme activity in the whole homogenate prepared from a liver sample that had been frozen previously (Table 2, Donor B) was substantially less than was the sum of the activity in the subcellular fractions obtained from a fresh liver sample from the same donor (Table 3, Donor B). This observation could be interpreted in several ways. One is that freezing causes some loss of enzyme activity.

In subsequent experiments, individual human liver aldehyde dehydrogenases were resolved chromatographically so that each could be examined for its ability to catalyze the oxidation of aldophosphamide and retinaldehyde. Frozen liver from Donor C was used as the starting material because of limited availability of fresh tissue. Subcellular fractionation into soluble and solubilized particulate fractions was not effected prior to column chromatography in the first part of this undertaking because preliminary isoelectric focusing experiments revealed that large amounts of enzymes known to localize in the particulate fraction were present in the soluble fraction prepared from frozen liver, *vide*

supra. Thus, liver tissue was homogenized directly in a 1.15% KCl solution containing 0.3% Lubrol® and 1 mM EDTA, pH 7.4; the homogenate was then submitted to successive ion exchange, affinity, and chromatofocusing column chromatography as described below. The substrates used to monitor the chromatographic separation included acetaldehyde, benzaldehyde, octanal, aldophosphamide, retinaldehyde, succinic semialdehyde, glutamic-γ-semialdehyde, γ-aminobutyraldehyde, and/or betaine aldehyde.

Chromatographic separation of human hepatic aldehyde dehydrogenases

DEAE-Sephacel chromatography of a Lubrol®-solubilized whole homogenate. A 105,000 *g* supernatant fraction prepared from a Lubrol®-treated liver whole homogenate was first submitted to DEAE-Sephacel column chromatography (Fig. 1A). Based on aldehyde dehydrogenase-catalyzed oxidation of the substrates used to monitor enzyme activity, the eluant was divided into seven pools.

Pools 1 and 2 each showed the highest aldehyde dehydrogenase activity with glutamic-γ-semialdehyde as the substrate (data not shown). Smaller amounts of activity were observed with succinic semialdehyde and aldophosphamide as substrates. No activity was detected in either pool with retinaldehyde as the substrate. Thirteen percent of the total recovered aldehyde dehydrogenase-catalyzed oxidation of aldophosphamide was by enzyme(s) present in Pools 1 and 2.

The enzyme(s) in Pool 3 showed a marked preference for succinic semialdehyde as the substrate; to a lesser extent, the oxidation of aldophosphamide and glutamic-γ-semialdehyde, but not that of retinaldehyde, was catalyzed by this pool. Approximately 17% of the total recovered aldehyde dehydrogenase-catalyzed oxidation of aldophosphamide was by enzyme(s) present in Pool 3.

Two closely spaced peaks of enzyme activity eluted next (Pools 4 and 5). The enzymes in each pool showed a marked preference for acetaldehyde as the substrate. These pools and the eluate in the gap between Pools 3 and 4 were the only ones that catalyzed the NAD-dependent oxidation of retinaldehyde; moreover, approximately 70% of the total recovered aldehyde dehydrogenase-catalyzed oxidation of aldophosphamide was by enzyme(s) present in these fractions. In the case of both aldphosphamide and retinaldehyde, the vast majority of the enzyme activity was present in Pool 4. Clearly, however, some was also present in Pool 5 although it could not be ascertained at this point whether this activity was due to the same enzyme(s) present in Pool 4 or to another enzyme. In the case of retinaldehyde, the former was suspected because there was no observable shoulder to the enzyme activity peak. In the case of aldophosphamide, the latter was thought to be the case because there was a clearly observable shoulder present.

Next to elute was a peak of enzyme activity for which betaine aldehyde was the preferred substrate (Pool 6). The enzyme(s) in this pool did not catalyze the oxidation of aldophosphamide or retinaldehyde.

A final peak of enzyme activity (acetaldehyde, octanal, benzaldehyde) was collected into Pool 7; the enzyme(s) in this pool did not catalyze the oxidation of either aldophosphamide or retinaldehyde.

Three "aldehyde dehydrogenases" found in human liver samples by others, viz. γ-aminobutyraldehyde dehydrogenase [14], ALDH-3 [22], and xanthine oxidase (dehydrogenase form) [23, 24], were not detected in this liver preparation. γ-Aminobutyraldehyde dehydrogenase-catalyzed oxidation of γ-aminobutyraldehyde (100 μM) was not observed when DEAE pools were assayed for this activity. ALDH-3 was not present as judged by the fact that no ALDH activity with a preference for benzaldehyde as substrate and NADP as cofactor was observed during chromatography on DEAE-Sephacel. Further, when the DEAE pools were submitted to isoelectric focusing, no ALDH activity (NADP/benzaldehyde) was observed in the pH range (5.9 to 6.4) where ALDH-3 is reported to focus [22]. Others have also observed that ALDH-3 is not expressed in all human liver specimens [25]. NAD-dependent xanthine oxidase-catalyzed oxidation of xanthine (50 μM) was not observed when the Lubrol®-solubilized whole homogenate was assayed for this activity. Xanthine oxidase activity has been observed previously to be lower in humans and carnivores than in rodents and herbivores [24], and to be lost during storage or upon freezing [12].

DEAE Pool 1, 2 *and* 3 *ALDH activity: Further resolution and purification, and enzyme identification.* Qualitatively similar profiles of enzyme activity were observed when DEAE Pools 1, 2, and 3 were subjected to affinity chromatography on Reactive Blue 2-Sepharose (Fig. 1, B, C, and D). The relative peak sizes differed but in all three cases an initial peak of enzyme activity with a marked preference for glutamic-γ-semialdehyde as the substrate eluted when a 25 mM sodium phosphate buffer, pH 7.4, was passed through the column, and a second peak of enzyme activity with a marked preference for succinic semialdehyde as the substrate eluted when this buffer was supplemented with 0.7 M NaCl and 1 mM NAD. Hence, these enzymes were identified as glutamic-γ-semialdehyde dehydrogenase, aka ALDH-4, and SSDH, respectively. Several additional observations supported these conclusions.

Upon isoelectric focusing, the enzyme identified as ALDH-4 resolved into two major bands with pI values of 6.7 and 6.9 (Table 4) which stained preferentially with glutamic-γ-semialdehyde as substrate. These pI values are characteristic of ALDH-4 [26]. Some additional lower pI bands were also observed; others have also observed these lower pI bands [26]. They noted that the presence and intensity of these bands varied with time, storage conditions, and repeated freezing and thawing. Finally, the K_m value of 238 μM for enzyme-catalyzed oxidation of glutamic-γ-semialdehyde (Table 4) is similar to the previously reported value of 170 μM for ALDH-4-catalyzed oxidation of this substrate [26].

The enzyme identified as SSDH was further purified with the aid of a 5′-AMP-Sepharose column (experiment not shown). No enzyme activity eluted when the column was washed with a 25 mM MES

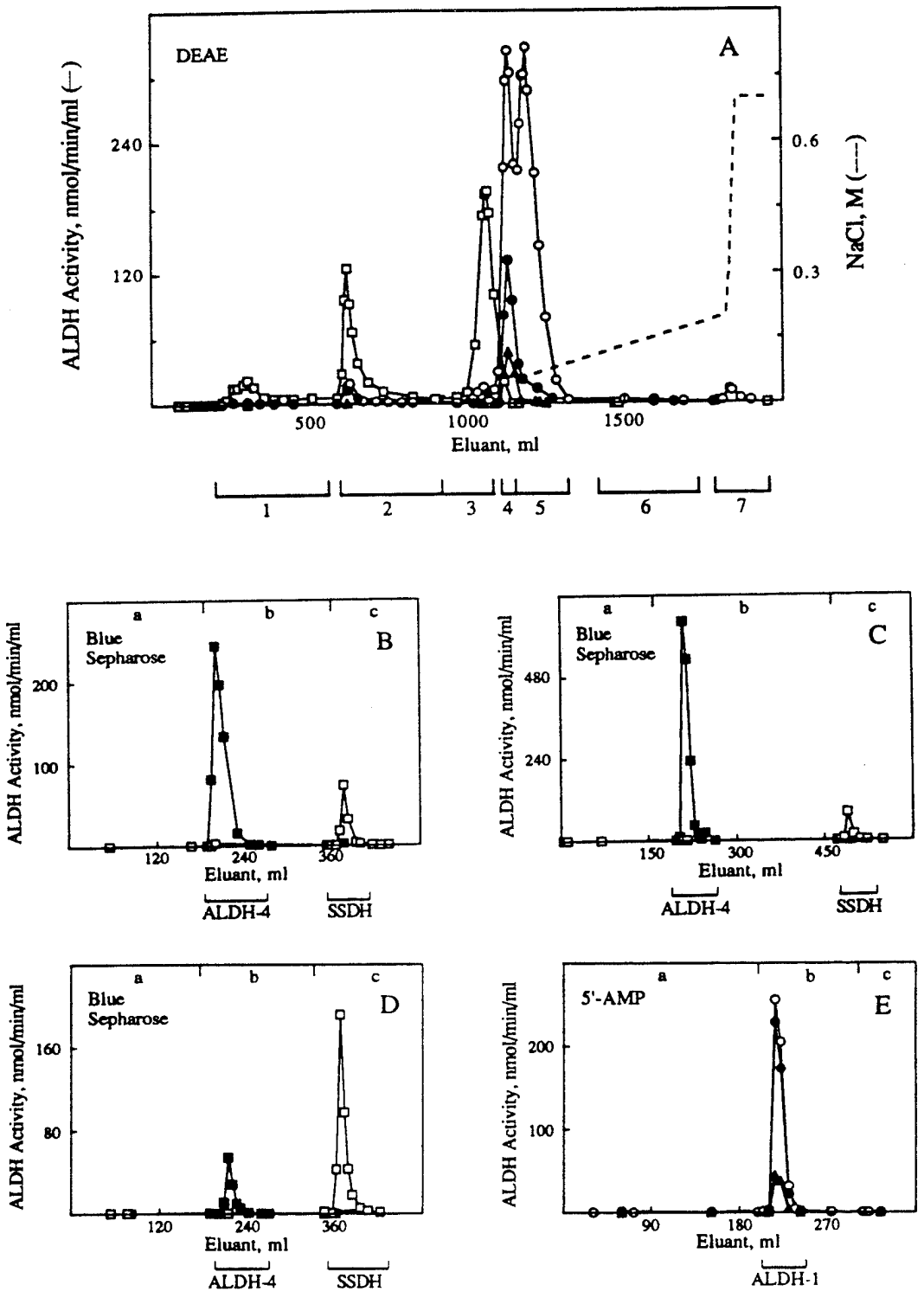

Fig. 1. Chromatographic separation of human liver aldehyde dehydrogenases. (A) A 105,000 *g* supernatant fraction obtained from a Lubrol®-treated whole homogenate prepared from a liver sample (6.7 g) originally obtained from Donor C and then frozen, was transferred, with the aid of Pharmacia PD-10 columns, from homogenization medium into a 20 mM imidazole buffer, pH 7.4 (buffer A). The latter preparation (110 mL) was loaded onto a DEAE-Sephacel column equilibrated with buffer A. The loaded column was eluted with 300 mL of buffer A, then with 470 mL of a 20 mM imidazole buffer, pH 6.8 (buffer B), and then with a 0 to 0.2 M NaCl gradient (900 mL) generated in buffer B. Elution was completed with 100 mL of buffer B adjusted to 0.7 M NaCl. Buffers A and B also contained 0.3% Lubrol®. (B) DEAE Pool 1 ALDH activity (400 mL) was concentrated 12-fold and then 28 mL of the concentrated pool was transferred into a 25 mM MES buffer, pH 6.6 (buffer C). This preparation was diluted to 95 mL with buffer C and then loaded onto a Reactive Blue 2-Sepharose column equilibrated with buffer C. The loaded column was developed as delineated at the *top* of the *panel*: (*a*) DEAE Pool 1 enzyme in buffer C followed by buffer C; (*b*) 25 mM sodium phosphate buffer, pH 7.4 (buffer D); and (*c*) buffer D containing 1 mM NAD and 0.7 M NaCl. (C) DEAE Pool 2 ALDH activity

Table 4. Isoelectric point and K_m values that were obtained and used to identify human liver aldehyde dehydrogenases

ALDH	Substrate	pI*	K_m† (μM)
1	Acetaldehyde	5.2	483
2	Acetaldehyde	4.9	<0.1
4	Glutamic-γ-semialdehyde‡	6.7, 6.9	238
5	Octanal	6.0–6.8	<0.1
BADH	Betaine aldehyde	6.5	0.3
SSDH	Succinic semialdehyde	6.0–7.5	<0.1

* Isoelectric point values were determined for each (semi)purified aldehyde dehydrogenase as described in Materials and Methods and in the legend to Fig. 2. Values are either from one determination or are the means of two to three determinations, each made on a separate gel.

† Aldehyde dehydrogenase activity was measured as described in Materials and Methods using the spectrophotometric assay. Double-reciprocal plots of initial rates (duplicate determinations) versus six to seven substrate concentrations were used to generate the kinetic constants for ALDHs 1 and 4; values are from single experiments. Integrated Michaelis equation analysis of single enzyme progress curves was used to generate the kinetic constants for ALDHs 2 and 5, and for succinic semialdehyde dehydrogenase and betaine aldehyde dehydrogenase; values are the means of three determinations.

‡ In aqueous solution, glutamic-γ-semialdehyde exists in equilibrium with its tautomer, DL-Δ^1-pyrroline-5-carboxylate. No attempt was made to differentiate between the tautomers, i.e. glutamic-γ-semialdehyde concentration was considered to be the total of glutamic-γ-semialdehyde and DL-Δ^1-pyrroline-5-carboxylate concentrations.

buffer, pH 6.6, or with the same buffer supplemented with 0.2 mM NAD. A single peak of enzyme activity (succinic semialdehyde) eluted when the column was subsequently washed with a 25 mM sodium phosphate buffer, pH 7.4, containing 0.7 M NaCl and 1 mM NAD. When visualized by either Coomassie Blue (protein) staining (data not shown) or by enzyme activity staining with succinic semialdehyde as substrate (Fig. 2), the enzyme thus obtained focused as several bands in the pH range 6.0 to 7.5. No bands appeared when glutamic-γ-semialdehyde was used as substrate in the enzyme activity stain. Multiple banding in the pH range of 6.3 to 7.2 has been reported for human brain SSDH [15]. Moreover, despite different assay conditions, the K_m value of <0.1 μM for enzyme-catalyzed oxidation of succinic semialdehyde (Table 4) is grossly similar to the previously reported values of 0.7 and 2 μM for human brain SSDH-catalyzed oxidation of this substrate [15].

SSDH catalyzed the oxidation of aldophosphamide, but ALDH-4 did not (Table 5). Neither catalyzed the oxidation of retinaldehyde (Table 5).

DEAE Pool 4 ALDH activity: Further purification,

Fig. 1. *Continued*
(325 mL) was concentrated 11-fold and 15 mL of the concentrated preparation was then transferred into buffer C. This preparation was diluted to 65 mL with buffer C and then loaded onto a Reactive Blue 2-Sepharose column equilibrated with buffer C. The loaded column was developed as delineated at the *top* of the *panel*: (*a*) DEAE Pool 2 enzyme in buffer C followed by buffer C; (*b*) buffer D; and (*c*) buffer D containing 1 mM NAD and 0.7 M NaCl. (D) DEAE Pool 3 ALDH activity (100 mL) was concentrated 5-fold and then transferred into buffer C. This preparation was diluted to 90 mL with buffer C and 80 mL of the diluted preparation was then loaded onto a Reactive Blue 2-Sepharose column equilibrated with buffer C. The loaded column was developed as delineated at the *top* of the *panel*: (*a*) DEAE Pool 3 enzyme in buffer C followed by buffer C; (*b*) buffer D; and (*c*) buffer D containing 1 mM NAD and 0.7 M NaCl. (E) DEAE Pool 4 ALDH activity (30 mL) was concentrated 3-fold and then transferred into buffer C. This preparation was diluted to 50 mL with buffer C and then loaded onto a 5′-AMP-Sepharose column equilibrated with buffer C. The loaded column was developed as delineated at the *top* of the *panel*: (*a*) DEAE Pool 4 enzyme in buffer C followed by buffer C; (*b*) 125 mM sodium phosphate buffer, pH 7.4 (buffer E) containing 0.7 mM NAD; and (*c*) buffer D containing 1 mM NAD and 0.7 M NaCl. In all panels, all buffers referred to also contained 1 mM dithiothreitol and 0.1 mM EDTA. Eluate was collected in fractions of 5–10 mL. Substrates used to monitor ALDH activity included 160 μM aldophosphamide (●), 5 μM retinaldehyde (▲), 4 mM acetaldehyde (○), 100 μM succinic semialdehyde (□) and 200 μM glutamic-γ-semialdehyde (■). Not all of the data obtained with these substrates is shown, nor is any of the data obtained when benzaldehyde (4 mM), octanal (100 μM), γ-aminobutyraldehyde (200 μM), or betaine aldehyde (50 μM) was used as the substrate.

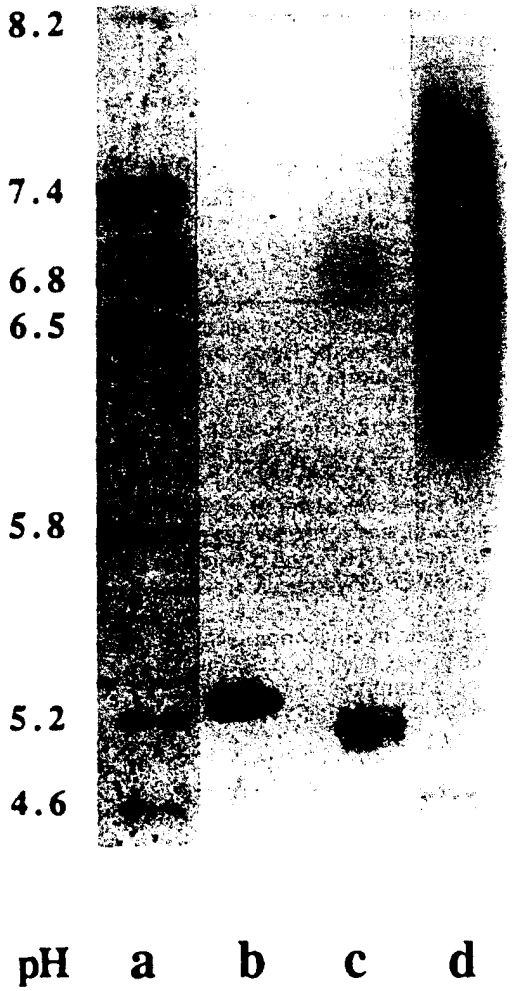

Fig. 2. Isoelectric focusing of relevant hepatic aldehyde dehydrogenases. ALDHs 1 and 2 (eluted from Polybuffer Exchanger columns), succinic semialdehyde dehydrogenase (eluted from a 5′-AMP-Sepharose column), and pI standards were subjected to isoelectric focusing. The amount of each enzyme loaded onto the gel was sufficient to generate 5–10 nmol NADH/min as determined by the spectrophotometric aldehyde dehydrogenase assay and the substrates with which the enzyme was ultimately stained. The nitroblue tetrazolium-based enzyme activity stain was used to visualize the aldehyde dehydrogenases present in lanes b, c and d. The aldehydes used as substrates were acetaldehyde (4 mM) for ALDHs 1 and 2, and succinic semialdehyde (100 μM) for SSDH. Coomassie Brilliant Blue was used to stain lane a. *Lane a*, pI standards; *lane b*, ALDH-1; *lane c*, ALDH-2; and *lane d*, SSDH. Additional details are presented in Materials and Methods.

and enzyme identification. DEAE Pool 4 ALDH activity was loaded onto a 5′-AMP-Sepharose column to which all of the aldehyde dehydrogenase activity bound (Fig. 1E). A single peak of enzyme activity eluted when a 125 mM sodium phosphate buffer, pH 7.4, supplemented with 0.7 mM NAD was subsequently passed through the column.

Several observations supported the identification of this enzyme as ALDH-1. After further purification by chromatofocusing chromatography (Fig. 3A), the enzyme was subjected to isoelectric focusing; a single band with a pI value of 5.2 was observed upon Coomassie Blue (protein) staining (data not shown) as well as upon enzyme activity staining with acetaldehyde as the substrate (Fig. 2 and Table 4). A pI value of 5.2 is characteristic of ALDH-1 [22] but is also not unlike that reported for γ-aminobutyraldehyde dehydrogenase [14]. However, γ-aminobutyraldehyde dehydrogenase focuses as a doublet; pI values are 5.3 and 5.45 [14]. Moreover, ALDH-1 is known to bind to 5′-AMP-Sepharose [21]. The enzyme in question bound to the 5′-AMP-Sepharose column; γ-aminobutyraldehyde dehydrogenase would not be expected to bind to 5′-AMP-Sepharose under the experimental conditions used [14]. Finally, the K_m value of 483 μM for enzyme-catalyzed oxidation of acetaldehyde (Table 4) is very similar to the previously reported value of 348 μM for ALDH-1 [27].

ALDH-1 catalyzed the oxidation of both aldophosphamide and retinaldehyde (Table 5).

DEAE Pool 5 ALDH activity: Further resolution and purification, and enzyme identification. Significant amounts of ALDH-1 were present in DEAE Pool 5. Preliminary experiments showed that resolution of ALDH-1 and the major enzyme in this pool by 5′-AMP-Sepharose and chromatofocusing chromatography was minimal. It was also noted in preliminary experiments with liver samples that had never been frozen, that the major aldehyde dehydrogenase present in DEAE Pool 5 was present in the 105,000 *g* pellet (particulate) fraction. ALDH-1 is a soluble enzyme. Therefore, in order to facilitate the purification of the major DEAE Pool 5 enzyme, it was decided to start with a preparation, viz. a Lubrol®-solubilized hepatic 105,000 *g* particulate fraction, that was virtually free of ALDH-1. The preparation was first submitted to DEAE-Sephacel column chromatography (Fig. 4A). A very minor peak of enzyme activity (acetaldehyde) eluted off the column first; it was closely followed by a major peak of enzyme activity (acetaldehyde). The last approximately two-thirds of the latter was collected into DEAE Pool 5′. DEAE Pool 5′ ALDH activity was then subjected to 5′-AMP-Sepharose affinity chromatography (Fig. 4B). Based on aldehyde dehydrogenase-catalyzed oxidation of the substrates used to monitor enzyme activity, the eluant was divided into three pools. The enzymes in all three pools showed a marked preference for acetaldehyde as substrate. As judged by isoelectric focusing, two aldehyde dehydrogenases were present in each pool, viz. a major band at pH 4.9 and a very minor band at pH 5.2 (data not shown). Pool 9 was further purified by chromatography on a Reactive Blue 2-Sepharose column (Fig. 4C). A trace of enzyme activity (acetaldehyde) eluted when a 25 mM MES buffer, pH 6.6, was passed through the column. A single peak of aldehyde dehydrogenase activity (acetaldehyde) eluted when the column was washed with a 125 mM phosphate buffer, pH 8.0, supplemented with 0.7 mM NAD.

Several observations supported the identification of this enzyme as ALDH-2. After further purification by chromatofocusing column chromatography (Fig. 3B), the enzyme was subjected to isoelectric focusing; a single band with a pI value of 4.9 was observed

Table 5. Data used to conclude that some human aldehyde dehydrogenases catalyze the oxidation of aldophosphamide and/or retinaldehyde whereas others do not

ALDH	Reference substrate*	Index value†	
		Aldophosphamide	Retinaldehyde
1	Acetaldehyde	64.0	9.8
2	Acetaldehyde	2.5	0.3[b]
4	Glutamic-γ-semialdehyde‡	0.04[a]	0.01[b]
5	Octanal	0.2[a]	0.04[b]
BADH	Betaine aldehyde	1.6[a]	0.06[b]
SSDH	Succinic semialdehyde	15.6	0.09[b]

* The indicated reference substrate for each enzyme was chosen as such because it was, or was one of, the preferred substrate(s) for that enzyme.

† Index value = (the observed, or smallest detectable, rate of aldophosphamide or retinaldehyde oxidation) (100)/(rate of oxidation of reference substrate). Values of 0.06(a) and 0.03(b) nmol/min were used to calculate the index values for aldophosphamide and retinaldehyde oxidation, respectively, when there was no measurable rate observed because each was considered to be the smallest detectable rate. Thus, they were used to calculate the upper limits of the relative rate of enzyme-catalyzed aldophosphamide and retinaldehyde oxidation that might be occurring but not be detected. The spectrophotometric assay was used to quantify aldehyde dehydrogenase-catalyzed oxidation of aldophosphamide (160 μM), acetaldehyde (4 mM), glutamic-γ-semialdehyde (200 μM), octanal (100 μM), betaine aldehyde (50 μM) and succinic semialdehyde (100 μM). The HPLC/spectrophotometric assay was used to quantify aldehyde dehydrogenase-catalyzed oxidation of retinaldehyde (25 μM).

‡ Glutamic-γ-semialdehyde concentration was calculated as described in Table 4.

upon Coomassie Blue (protein) staining (data not shown), as well as upon enzyme activity staining using acetaldehyde as the substrate (Fig. 2 and Table 4). This value is characteristic of ALDH-2 [22]. Despite the difference in assay consitions, the low K_m value of <0.1 μM for enzyme-catalyzed oxidation of acetaldehyde (Table 4) is grossly similar to the previously reported values of <1 μM [28] and 2.4 and 3 μM [21], for ALDH-2-catalyzed oxidation of this substrate.

ALDH-2 catalyzed the oxidation of aldophosphamide but not that of retinaldehyde (Table 5).

DEAE Pool 6 ALDH activity: Further resolution and purification, and enzyme identification. 5′-AMP-Sepharose column chromatography was used to further resolve and purify DEAE Pool 6 ALDH activity (data not shown). An initial, small peak of enzyme activity with a marked preference for acetaldehyde eluted when a 25 mM sodium phosphate buffer, pH 7.4, was passed through the column. A second peak of enzyme activity with a marked preference for betaine aldehyde eluted when a 125 mM sodium phosphate buffer, pH 7.4, was subsequently passed through the column.

On the basis of substrate preference and isoelectric focusing point, viz. 4.9, the first enzyme to elute was identified as ALDH-2.

Several observations supported the identification of the second enzyme to elute as BADH. It focused as a single band at a pI value of 6.5, which stained most intensely when betaine aldehyde was used as the substrate (Table 4). Additionally, the K_m value of 0.3 μM for enzyme-catalyzed oxidation of betaine aldehyde (Table 4) is grossly similar to the previously reported value of 10 μM for oxidation of this substrate by BADH [13].

BADH did not catalyze the oxidation of aldophosphamide or retinaldehyde (Table 5).

DEAE Pool 7 ALDH activity: Enzyme identification. The aldehyde dehydrogenase present in DEAE Pool 7 showed a marked preference for aromatic and long chain aliphatic aldehydes. It was identified as ALDH-5 on the basis of several criteria. First, it focused as a diffuse smear extending from pH 5.5 to 7.5 with most of the enzyme activity appearing between pH 6.0 and 6.8 (Table 4). These values are consistent with those previously reported for ALDH-5 [12]. Second, the K_m value for oxidation of acetaldehyde by the enzyme in DEAE Pool 7 was in the millimolar range (data not shown) while the K_m value of <0.1 μM for the oxidation of octanal by this enzyme was much lower (Table 4). These values are consistent with the reports that ALDH-5 shows a preference for long chain aldehydes as substrates [12, 13].

ALDH-5 did not catalyze the oxidation of either aldophosphamide or retinaldehyde (Table 5).

Michaelis–Menten kinetics and proportional importance of individual hepatic aldehyde dehydrogenases that catalyze the oxidation of aldophosphamide and retinaldehyde

During the chromatographic separation of the hepatic aldehyde dehydrogenases, careful records were kept regarding the fraction of the total hepatic aldehyde dehydrogenase activity (160 μM aldophosphamide, 25 μM retinaldehyde) that partitioned with each aldehyde dehydrogenase; these values are presented in Table 6 under "%

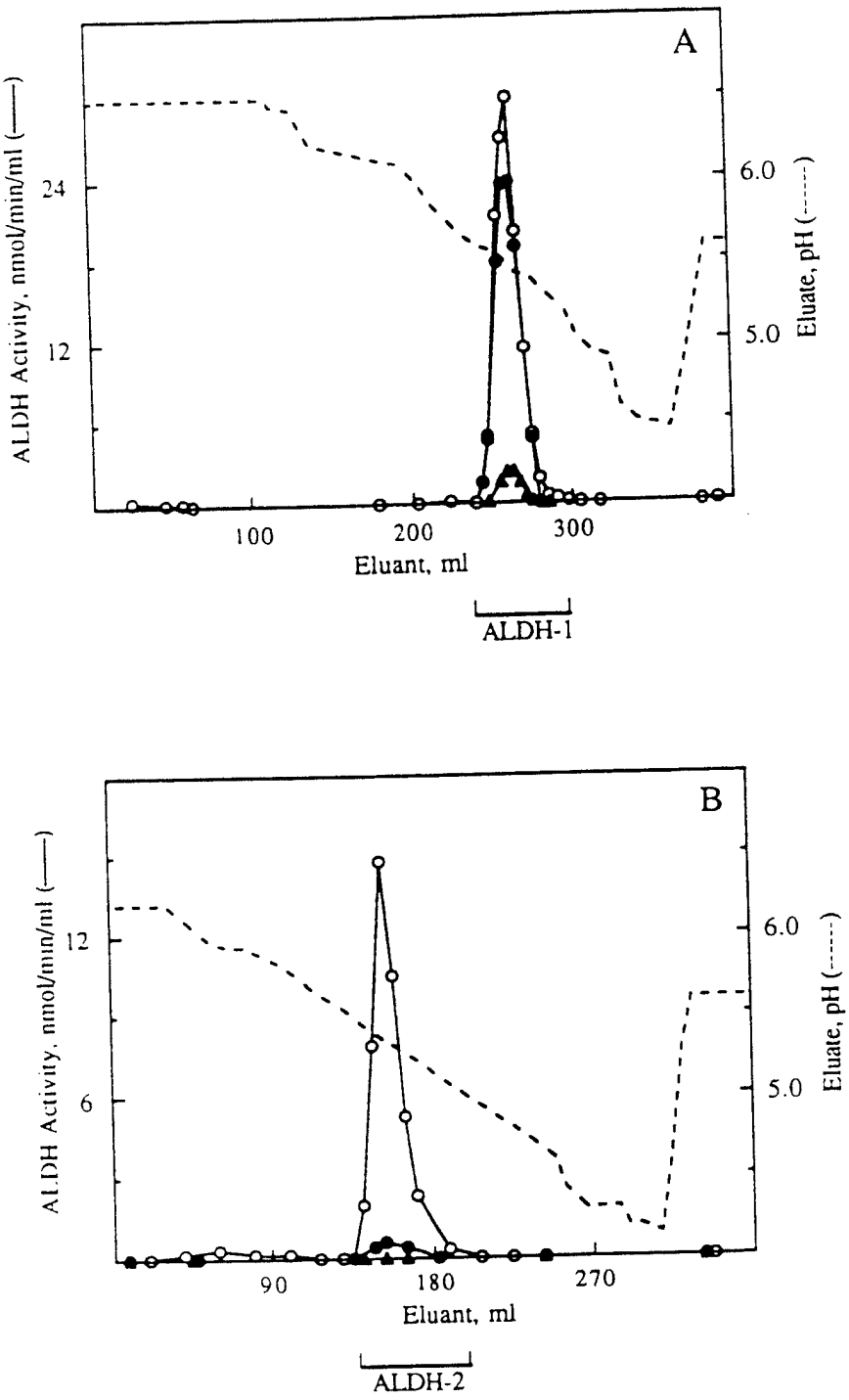

Fig. 3. Chromatofocusing of ALDH-1 and ALDH-2. (A) ALDH-1 recovered from the 5'-AMP-Sepharose column (Fig. 1E) was concentrated 4-fold and transferred, with the aid of PD-10 columns, into a 25 mM histidine buffer, pH 6.5 (buffer F). This preparation (18 mL) was diluted to 100 mL with buffer F and was loaded onto a column of Polybuffer Exchanger 94 that had been equilibrated with buffer F. The column was developed with 250 mL of 10% Polybuffer 74, pH 4.5, followed by 50 mL of this same buffer containing 0.7 M NaCl. The elution maximum was at pH 5.5. (B) ALDH-2 recovered from the Reactive Blue 2-Sepharose column (Fig. 4C) was concentrated 8-fold and transferred into a 25 mM histidine buffer, pH 6.2 (buffer G). This preparation (13 mL) was loaded onto a column of Polybuffer Exchanger 94 that had been equilibrated with buffer G. The column was developed with 300 mL of 10% Polybuffer 74, pH 4.0, followed by 50 mL of this same buffer containing 0.7 M NaCl. The elution maximum was at pH 5.3. In both panels, all buffers referred to also contained 1 mM dithiothreitol, 0.1 mM EDTA, and 20% glycerol. Eluate was collected in fractions of 2–8 mL. Substrates used to monitor ALDH activity were 160 μM aldophosphamide (●), 5 μM retinaldehyde (▲) and 4 mM acetaldehyde (○).

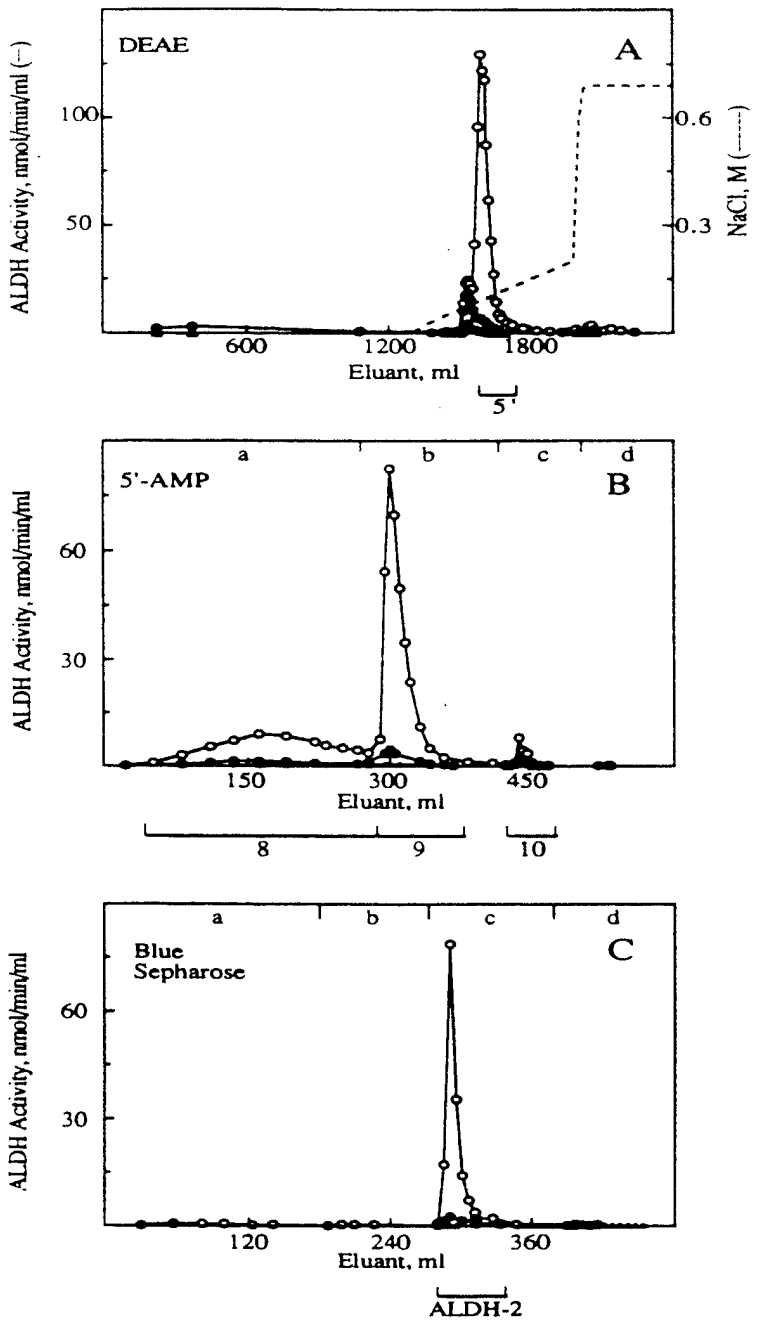

Fig. 4. Chromatographic purification of human liver ALDH-2. (A) A Lubrol®-solubilized 105,000 *g* particulate fraction prepared from a liver sample (15.5 g) originally obtained from Donor C and then frozen, was transferred, with the aid of PD-10 columns, into a 20 mM imidazole buffer, pH 7.4 (buffer A). The latter preparation (340 mL) was loaded onto a DEAE-Sephacel column equilibrated with buffer A. The loaded column was eluted with 550 mL of buffer A, then with 450 mL of a 20 mM imidazole buffer, pH 6.8 (buffer B), and then with a 0–0.2 M NaCl gradient (800 mL) generated in buffer B. Elution was completed with 300 mL of buffer B adjusted to 0.7 M NaCl. (B) DEAE Pool 5' ALDH activity was concentrated 7-fold and then transferred into a 25 mM MES buffer, pH 6.6 (buffer C). The latter preparation was diluted to 140 mL with buffer C and was then loaded onto a 5'-AMP-Sepharose column equilibrated with the same buffer. The loaded column was developed as delineated at the *top* of the *panel*: (*a*) DEAE Pool 5' enzyme in buffer C followed by buffer C; (*b*) 125 mM sodium phosphate buffer, pH 8.0 (buffer H); (*c*) buffer H containing 0.7 mM NAD; and (*d*) 25 mM sodium phosphate buffer, pH 7.4 (buffer D) containing 1 mM NAD and 0.7 M NaCl. (C) Pool 9 from the 5'-AMP column was concentrated 19-fold and then transferred into buffer C. The latter preparation was diluted to 50 mL and was then loaded onto a Reactive Blue 2-Sepharose column equilibrated with the same buffer. The loaded column was developed as delineated at the *top* of the *panel*: (*a*) 5'-AMP Pool 2 enzyme in buffer C followed by buffer C; (*b*) buffer H; (*c*) buffer H containing 0.7 mM NAD; and (*d*) buffer D containing 1 mM NAD and 0.7 M NaCl. In all panels, all buffers referred to also contained 1 mM dithiothreitol and 0.1 mM EDTA. Eluate was collected in fractions of 5–10 mL. Substrates used to monitor ALDH activity were 160 μM aldophosphamide (●), 5 μM retinaldehyde (▲) and 4 mM acetaldehyde (○).

Table 6. Human liver aldehyde dehydrogenases: Kinetics of, and relative contribution to, the catalytic oxidation of aldophosphamide and retinaldehyde*

	Aldophosphamide			Retinaldehyde		
ALDH	K_m (μM)	V_{max}† (μmol/min/mg protein)	% Contribution‡	K_m (μM)	V_{max}† (μmol/min/mg protein)	% Contribution§
1	52 ± 3	4.8 ± 0.1	64	0.3 ± 0.1	0.5 ± 0.1	100
2	1193 ± 110	0.4 ± 0.0	6	NS	0	0
4	NS‖	0	0	NS	0	0
5	NS	0	0	NS	0	0
SSDH	560 ± 48	0.9 ± 0.1	30	NS	0	0
BADH	NS	0	0	NS	0	0

* Double-reciprocal plots of initial rates versus substrate concentrations were constructed as described in Materials and Methods and in the legend to Fig. 5 to estimate the K_m (±SEM) and V_{max} (±SEM) values for enzyme-catalyzed oxidation of aldophosphamide. Values and error estimates are from single experiments (duplicate individual initial rate measurements made at each of four to ten substrate concentrations in each case). The integrated Michaelis method of analysis of single enzyme progress curves (N = 4) was used as described in Materials and Methods to generate the K_m (±SEM) and V_{max} (±SEM) values for ALDH-1-catalyzed oxidation of retinaldehyde.

† ALDHs 1 and 2, purified through chromatofocusing column chromatography, and SSDH, purified through 5'-AMP-Sepharose column chromatography, were used to determine V_{max} values for aldophosphamide and, where indicated, for retinaldehyde, oxidation.

‡ Percentage of total recovered hepatic aldehyde dehydrogenase activity measurable with 160 μM aldophosphamide that co-purifies with the indicated aldehyde dehydrogenase during a typical purification.

§ Percentage of total recovered hepatic aldehyde dehydrogenase activity measurable with 25 μM retinaldehyde that co-purifies with the indicated aldehyde dehydrogenase during a typical purification.

‖ NS: not a substrate.

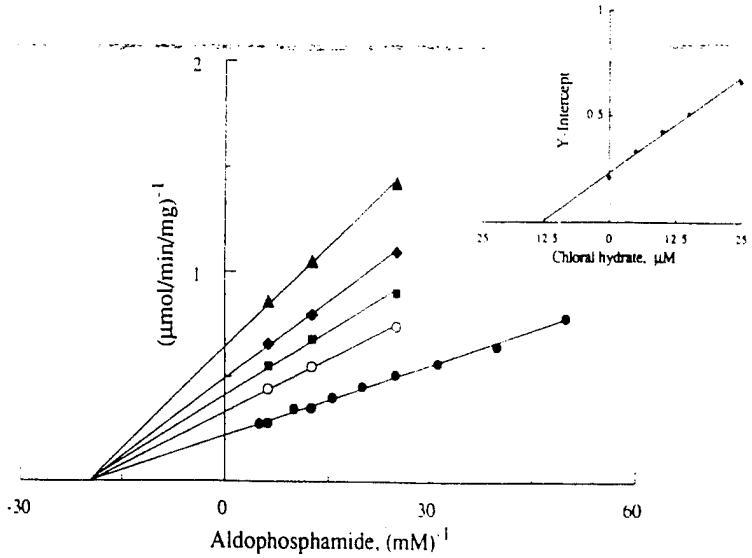

Fig. 5. Inhibition of ALDH-1-catalyzed oxidation of aldophosphamide by chloral hydrate: Lineweaver–Burk kinetic analysis. Initial rates of ALDH-1 catalyzed aldophosphamide oxidation were measured as described in Materials and Methods. The concentrations of chloral hydrate were 0 (●), 5 (○), 10 (■), 15(◆) and 25 (▲) μM. ALDH-1 purified through chromatofocusing chromatography was used in this experiment. V_{max} and K_m values were calculated from the y- and x-intercepts, respectively, of the fitted lines and were 4.8 μmol/min/mg and 52 μM, respectively. Data points represent the means of duplicate determinations. The K_i value was calculated from the plot of the y-intercept values against the chloral hydrate concentrations (inset) and was 13 μM.

Contribution". Three enzymes, viz. ALDH-1, ALDH-2, and SSDH, catalyzed the oxidation of aldophosphamide; ALDH-1 was by far the most important, accounting for 64% of the total when the aldophosphamide concentration was 160 μM. Given the K_m values obtained, ALDH-1 would account for an even greater percentage of the total when the aldophosphamide concentration was pharmacological. K_m values for the catalysis of aldophosphamide by ALDH-1, ALDH-2, and SSDH were 52, 1193 and 560 μM, respectively (Table 6). The Lineweaver-Burk plot used to generate the K_m and V_{max} values for ALDH-1-catalyzed oxidation of aldophosphamide is shown in Fig. 5. ALDH-1 accounted for all of the recovered NAD-dependent enzyme-catalyzed oxidation of retinaldehyde. The K_m value for ALDH-1-catalyzed oxidation of retinaldehyde was 0.3 μM (Table 6).

Sensitivity of ALDH-1-, ALDH-2- and SSDH-catalyzed oxidation of aldophosphamide to inhibition by chloral hydrate

Detoxification of aldophosphamide by human pluripotent hematopoietic progenitor cells appears to be catalyzed by an aldehyde dehydrogenase relatively insensitive to inhibition by chloral hydrate [29]. Therefore, the sensitivity of the three human aldehyde dehydrogenases known to catalyze the oxidation of aldophosphamide, to inhibition by chloral hydrate was determined. ALDH-1-catalyzed oxidation of aldophosphamide was highly sensitive to inhibition by chloral hydrate, whereas ALDH-2- and SSDH-catalyzed oxidation of this substrate was less sensitive (Table 7). The K_i value for chloral hydrate inhibition of ALDH-1-catalyzed oxidation of aldophosphamide was 13 μM; inhibition was noncompetitive (Fig. 5).

Table 7. Inhibition of aldehyde dehydrogenase-catalyzed aldophosphamide oxidation by chloral hydrate

	Inhibition by chloral hydrate† (%)	
Enzyme*	0.1 mM	1.0 mM
ALDH-1	82	97
ALDH-2	24	51
SSDH	6	16

* ALDHs 1 and 2 were the chromatofocused preparations. SSDH was semipurified from DEAE Peak 3 by passage though Blue Sepharose and 5'-AMP affinity columns. For additional details, see Materials and Methods and the text of Results.

† Initial rates of aldehyde dehydrogenase-catalyzed aldophosphamide (160 μM) oxidation were measured in the presence and absence of chloral hydrate as described in Materials and Methods. Control rates (no chloral hydrate) were 6.1, 0.13 and 2.0 nmol/min for ALDH-1, ALDH-2 and SSDH, respectively.

DISCUSSION

Six aldehyde dehydrogenases were resolved from human liver. One, viz. ALDH-1, accounted for all

of the NAD-dependent enzyme-catalyzed oxidation of retinaldehyde in this organ. Three aldehyde dehydrogenases, viz. ALDH-1, ALDH-2, and SSDH, catalyzed the oxidation of aldophosphamide. ALDH-1 was, by far, the most efficient in this regard. It is not the hepatic aldehyde dehydrogenase that is most important in catalyzing the oxidation of acetaldehyde. That distinction goes to ALDH-2 [30–32]. ALDH-4, ALDH-5, and BADH did not catalyze the oxidation of either aldophosphamide or retinaldehyde.

These observations closely parallel those made in mouse liver. Thus, *in vitro*, AHD-2, the mouse homolog of ALDH-1, catalyzes the bulk of the oxidation of both aldophosphamide and retinaldehyde in mouse liver; AHD-5 and AHD-12, the mouse homologs of ALDH-2 and SSDH, respectively, catalyze the oxidation of aldophosphamide but not that of retinaldehyde, and AHD-3, the mouse homolog of ALDH-5, does not catalyze the oxidation of aldophosphamide or retinaldehyde [8, 9].

There are some differences. Thus, mouse AHDs-1 and -9, unlike their human counterparts, ALDH-4 and BADH, respectively, contribute to the overall hepatic oxidation of aldophosphamide [9]. Further, human homologs of mouse AHDs -7, -8, -10, -11 and -13 were not found. AHD-7 catalyzes the oxidation of retinaldehyde [8], but not that of aldophosphamide [9]. AHDs -8, -10, -11, and -13 catalyze the oxidation of aldophosphamide [9], but not that of retinaldehyde [8]. Failure to find homologs of these enzymes in the human liver sample examined may be due to species differences and/or to loss of activity upon storage and freezing of the human liver sample.

It was not possible to determine whether xanthine oxidase (dehydrogenase form), ALDH-3, or γ-aminobutyraldehyde dehydrogenase catalyzed the oxidation of either retinaldehyde or aldophosphamide because these enzymes were not found in the liver sample used in our investigation. The dehydrogenase form of mouse xanthine oxidase catalyzes the oxidation of retinaldehyde but not that of aldophosphamide [8, 9]. Perhaps the same is true of the human homolog. AHD-4, an aldehyde dehydrogenase found in mouse stomach but not in mouse liver [10], catalyzes, albeit relatively poorly, the oxidation of aldophosphamide but not that of retinaldehyde [8, 9]. ALDH-3 may be the human homolog of this enzyme [11]. In that event, it may catalyze the oxidation of aldophosphamide but not that of retinaldehyde.

Oxazaphosphorines such as cyclophosphamide are detoxified when aldophosphamide is oxidized to carboxyphosphamide [3]. Depending on the type of neoplasm, the oxazaphosphorines often exhibit a relatively favorable therapeutic index [3]. At least in part, this is because certain critical and rapidly renewing normal cell populations, e.g. pluripotent hematopoietic, and intestinal epithelial, progenitor cells contain an aldehyde dehydrogenase which catalyzes the relevant detoxification whereas some tumor cell populations lack such an enzyme or contain much less of it [33–39]. The identity of the relevant aldehyde dehydrogenases in any given normal tissue is, at present, largely uncertain. Whereas the expression of aldehyde dehydrogenase activity of some sort is nearly ubiquitous in normal tissues [40], the expression of any particular aldehyde dehydrogenase is more restricted. Thus, while high levels of ALDH-1 and ALDH-2 are found in liver and kidney, much lower levels are found in intestine, brain, stomach, lung, spleen, testis, lymphocytes, scalp skin, hair roots, cornea and placenta [2, 12, 22, 41, 42]. ALDH-1 is apparently not present in heart [12, 22] and is the only aldehyde dehydrogenase present in erythrocytes [43, 44]. The tissue distribution of SSDH is less clear; to date it has been identified in brain [15] and liver.

ALDH-1 would be expected to confer protection against oxazaphosphorines on any cell in which it is expressed. Conversely, cells which lack ALDH-1, e.g. those of the heart [12, 22], might be expected to be at increased risk to the toxic actions of oxazaphosphorines. Perhaps related is the severe cardiotoxicity observed when high doses of cyclophosphamide are given [45]. Individuals who express a relatively enzymatically nonfunctional variant of ALDH-1 [46, 47] might be at increased risk to the untoward systemic actions of cyclophosphamide and other oxazaphosphorines.

As judged by the kinetic values obtained, ALDH-2, even when present in large amounts, is highly unlikely to confer significant protection on any tissue. Consequently, the large (up to 50%) fraction of Orientals that express an enzymatically nonfunctional variant of this aldehyde dehydrogenase [48] are unlikely to be at increased risk to the untoward systemic actions of the oxazaphosphorines.

SSDH may be an important contributor to detoxification of the oxazaphosphorines in tissues which do not express ALDH-1.

It has been demonstrated that aldehyde dehydrogenase-catalyzed detoxification of aldophosphamide contributes significantly to the relative insensitivity of murine intestinal crypt cells [49] and murine and human hematopoietic progenitor cells to the oxazaphosphorines [33–37]. The aldehyde dehydrogenase present in murine intestinal crypt cells is kinetically and immunologically identical to the major liver cytosolic aldehyde dehydrogenase, viz. AHD-2 [49]. Uncertain is the identity of the operative aldehyde dehydrogenase(s) in hematopoietic progenitor cells. The experiments of Kastan and coworkers [39] suggest that in humans it is ALDH-1 because polyclonal antibodies that they raised to a cytoplasmic (most probably cytosolic) aldehyde dehydrogenase (most probably ALDH-1) purified from Hep G2 hepatoma recognized a protein (presumably ALDH-1) present in hematopoietic progenitor cells (identified as such by flow cytometric assay of cell surface lineage- and maturation-specific antigens). The polyclonal antibodies used to identify the hematopoietic progenitor cell protein also recognized an approximately 55 kDa protein in a cytosolic extract of human liver [39]. Both ALDH-1 and ALDH-2 are tetramers made up of approximately 55 kDa subunits [21]. The specificity of the polyclonal antibodies used to identify the proteins (presumably aldehyde dehydrogenases) in these experiments was not reported. Inconsistent

with the notion that the operative enzyme is ALDH-1 is the observation that even high concentrations of chloral hydrate, a potent inhibitor of human ALDH-1 and mouse AHD-2, did not, or only minimally, potentiate(d) the cytotoxic action of the oxazaphosphorines against multipotent hematopoietic progenitor cells whereas other known inhibitors of these enzymes did [29]. The operative enzyme could be SSDH. It is relatively insensitive to inhibition by chloral hydrate. However, SSDH is a particulate enzyme and, in experiments conducted by yet another laboratory [15], antisera raised to ALDH-1 did not recognize brain SSDH; further, the subunit size of brain SSDH is approximately 62 kDa.

Expression of AHD-2, the mouse homolog of ALDH-1, is a demonstrated mechanism of acquired resistance to the oxazaphosphorines in two mouse leukemia lines [50–52], and increased expression of an unidentified aldehyde dehydrogenase appears to be the underlying basis for the acquired resistance to oxazaphosphorines observed in a rat leukemia [53]. Elevated aldehyde dehydrogenase expression may be a clinically important mechanism for acquired resistant to the oxazaphosphorines in human tumors. In cases of intrinsic and/or acquired tumor cell resistance to the oxazaphosphorines where aldehyde dehydrogenase-catalyzed detoxification of aldophosphamide is the causative event, therapeutic intervention strategies based on selective sensitization of the resistant tumor cell population may be possible if the operative tumor enzyme differs from that present in critical normal cell populations since aldehyde dehydrogenases often differ in their relative sensitivity to various inhibitors, e.g. chloral hydrate [54–57]. On the other hand, given that aldehyde dehydrogenase-catalyzed detoxification of aldophosphamide serves to protect certain critical normal cell populations against the cytotoxic action of oxazaphosphorines, *vide supra*, and that a number of frequently used drugs, e.g. certain cephalosporins, are known to inhibit at least some of the aldehyde dehydrogenases [58, 59], the potential for clinically adverse drug interactions is apparent. Further, given that ALDH-1 activity in erythrocytes and liver is reduced in chronic alcoholics [42, 60–62], and that aldehyde dehydrogenase activity is apparently depressed in humans given certain oral contraceptives [63], these populations may be at increased risk of oxazaphosphorine-induced systemic toxicities. Also, induction of aldehyde dehydrogenase activity is a demonstrated phenomenon in rodents [64, 65].

Retinoids, a family of natural and synthetic vitamin A analogs, are recognized as important regulators of the growth and differentiation of normal and transformed cells [66]. Since retinoic acid is the most potent of the naturally occurring retinoids in many of the systems used to assay retinoids for such activity [66–68], retinol is the major retinoid in the circulation [69], and oxidation of retinol to retinoic acid proceeds via the intermediate, retinaldehyde [7, 70, 71], enzyme-catalyzed oxidation of retinaldehyde to retinoic acid can be viewed as a bioactivation and most likely occurs in target cells. Thus, the rate and extent of retinoid-mediated differentiation of cells could very well be influenced by the rate at which cells convert retinol to retinoic acid. Supporting this notion is the observation that conversion of retinaldehyde to retinoic acid in keratinocytes undergoing differentiation occurs at a greater rate than in keratinocytes that are not [7].

Aldehyde dehydrogenases, aldehyde oxidase and xanthine oxidase have all been reported to catalyze the oxidation of retinaldehyde to retinoic acid [5, 6, 8, 72–77]. Available evidence suggests that, at least in some tissues, retinaldehyde oxidation is catalyzed primarily by one or more of the cytosolic, NAD-dependent, disulfiram-sensitive, substrate nonspecific, aldehyde dehydrogenases [5–8, 73, 76].

Of the six human hepatic aldehyde dehydrogenases examined in the present investigation, only ALDH-1 catalyzed the oxidation of retinaldehyde to retinoic acid. In addition, a retinaldehyde dehydrogenase apparently distinct from ALDH-1 has been identified in human keratinocytes [7]. The low K_m value for ALDH-1-catalyzed oxidation of retinaldehyde to retinoic acid is consistent with a physiological role for ALDH-1 in the *in vivo* generation of retinoic acid in those tissues where it is present.

Many neoplastic cell populations apparently lack ALDH-1 or, in species other than humans, its homolog [36, 38, 51]. This may preclude their ability to generate retinoic acid from retinaldehyde and, thus, to respond to retinol and β-carotene, another precursor of retinaldehyde [70, 71, 77, 78]. Further, relevant genetic polymorphism, and modulation of ALDH-1 activity, may have profound effects on retinoic acid-dependent processes.

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Transient changes in tumor cell sensitivity to various anticancer drugs induced by frequently ingested dietary constituents. Rekha, G. K. and Sladek, N. E., Dept. of Pharmacology, University of Minnesota, Minneapolis, MN 55455.

Determinants of cellular sensitivity to anticancer drugs include enzymes that catalyze their biotransformation. For example, cytosolic class-3 aldehyde dehydrogenase, glutathione S-transferase and UDP-glucuronosyl transferase are known/thought to catalyze the bioinactivation of oxazaphosphorines [e.g., cyclophosphamide and mafosfamide (MAF)], melphalan (MEL) and mitoxantrone (MIT), respectively, and cytochrome P450 IA1 and DT-diaphorase are known/thought to catalyze the bioactivation of ellipticine (ELP) and the indoloquinone EO9, respectively. Brief exposure to any one of a number of abundantly and widely present dietary/environmental agents markedly, coordinately and transiently induces some or all of the aforementioned enzymes. Thus, methylcholanthrene (MC) induces each of these enzymes in the model system that we use, viz., cultured human breast adenocarcinoma MCF-7/0 cells, and all but P450 IA1 are induced after exposure to catechol (CAT). Given the foregoing, the expectations were that 1) MCF-7/0 cells exposed to MC or CAT would become transiently more sensitive to EO9 and transiently more insensitive to MAF, MEL and MIT, and 2) MCF-7/0 cells exposed to MC, but not those exposed to CAT, would become transiently more sensitive to ELP. Each of these expectations was realized. Extrapolated to the clinic, these observations strongly indicate that, if an optimally effective cancer chemotherapeutic strategy is to be achieved, consideration must be given to not only the drug(s) to be used, but also, to the diet that will enable the selected drug(s) to be maximally effective. Supported by USPHS CA 21737 and DOD DAMD 17-94-J-4057.

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EXPERIMENTAL THERAPEUTICS

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Cytosolic class-3 and class-1 aldehyde dehydrogenase activities (ALDH-3 and ALDH-1, respectively) in human primary breast tumors. Sladek, N. E. and Sreerama, L., Dept. of Pharmacology, U. of Minnesota, Mpls., MN 55455.

Cultured rodent and human tumor cell lines have been used previously to establish that cellular sensitivity to oxazaphosphorines such as cyclophosphamide and mafosfamide decreases as cellular content of ALDH-3 and/or ALDH-1 increases. Not known is the extent of interindividual variation, if any, in the activity of either of these enzymes for a given tumor type. Thus, we have begun to quantify ALDH-3 and ALDH-1 activities in human primary tumors, specifically in breast tumors because cyclophosphamide has long been, and continues to be, the cornerstone of breast cancer chemotherapy. ALDH-3 activity (NADP-dependent catalysis of benzaldehyde oxidation) in human breast tumors (n = 20) ranged from 2 to 247 mIU/g tissue; mean and median values were 22 and 10 mIU/g tissue, respectively. ALDH-3 activity in human normal breast tissue (n = 7) ranged from 2 to 15 mIU/g tissue; mean and median values were 8 and 8 mIU/g tissue, respectively. ALDH-1 activity (NAD-dependent catalysis of acetaldehyde oxidation) in the breast tumors ranged from 4 to 57 mIU/g tissue; mean and median were 24 and 18 mIU/g tissue, respectively. ALDH-1 activity in normal breast tissue ranged from 5 to 21 mIU/g tissue; mean and median values were 11 and 16 mIU/g tissue, respectively. Given the wide range of ALDH-3 and ALDH-1 activities that were observed, measurement of these enzyme levels is likely to be of value with regard to predicting the therapeutic value, or lack thereof, of oxazaphosphorine therapy, at least in the case of breast cancer. Supported by USPHS Grant CA 21737 and DOA DAMD17-94-J-4057.

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IDENTIFICATION AND CHARACTERIZATION OF A NOVEL CLASS 3 ALDEHYDE DEHYDROGENASE OVEREXPRESSED IN A HUMAN BREAST ADENOCARCINOMA CELL LINE EXHIBITING OXAZAPHOSPHORINE-SPECIFIC ACQUIRED RESISTANCE*

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Abstract—Associated with the oxazaphosphorine-specific acquired resistance exhibited by a human breast adenocarcinoma subline growing in monolayer culture, viz. MCF-7/OAP, was the overexpression (>100-fold as compared with the very small amount expressed in the oxazaphosphorine-sensitive parent line) of a class 3 aldehyde dehydrogenase, viz. ALDH-3, judged to be so because it is a polymorphic enzyme (pI values *ca.* 6.0) present in the cytosol that is heat labile, is insensitive to inhibition by disulfiram (25 μM), much prefers benzaldehyde to acetaldehyde as a substrate and, at concentrations of 4 mM, prefers NADP to NAD as a cofactor. No other aldehyde dehydrogenases were found in these cells. As compared with those of the prototypical class 3 human ALDH-3, viz. constitutive human stomach mucosa ALDH-3, the physical and catalytic properties of the MCF-7/OAP enzyme differed somewhat with regard to pI values, native M_r, subunit M_r, recognition of the subunit by anti-stomach ALDH-3 IgY, pH stability, cofactor influence on catalytic activity, and the ability to catalyze, albeit poorly, the oxidation of an oxazaphosphorine, viz. aldophosphamide. Hence, the MCF-7/OAP ALDH-3 was judged to be a novel class 3 aldehyde dehydrogenase. Small amounts of a seemingly identical enzyme are also present in normal pre- and post-menopausal breast tissue. None could be detected in human liver, kidney or placenta, suggesting that it may be a tissue-specific enzyme.

Mafosfamide, 4-hydroperoxycyclophosphamide and cyclophosphamide are antineoplastic agents collectively referred to as oxazaphosphorines [1]. Each is a prodrug, i.e. *per se*, without therapeutic (cytotoxic) activity. Salient aspects of their metabolism are presented in Fig. 1. Most pertinent to the present investigation is the irreversible detoxification that occurs when NAD(P)-dependent aldehyde dehydrogenases catalyze the oxidation of a pivotal metabolite, viz. aldophosphamide, to carboxyphosphamide. Class 1 aldehyde dehydrogenases, e.g. human ALDH‡-1, are particularly important in this regard [2, 3]. At least two other human "aldehyde" dehydrogenases, viz. ALDH-2 (a class 2 aldehyde dehydrogenase) and succinic semialdehyde dehydrogenase (SSDH), also catalyze the reaction albeit less well; still others, viz. ALDH-4 (glutamic γ-semialdehyde dehydrogenase), ALDH-5 (a microsomal class 3 aldehyde dehydrogenase) and betaine aldehyde dehydrogenase (BADH), do not catalyze it at all [3]. Not known is whether a cytosolic class 3 aldehyde dehydrogenase, viz. ALDH-3, catalyzes the reaction but a putative homolog of it, viz. mouse AHD-4, is known to do so [2]; large amounts of ALDH-3 are found in human stomach mucosa though not in the liver [4].

Relatively elevated levels of a relevant aldehyde dehydrogenase would be the basis of a relatively decreased cellular sensitivity to the oxazaphosphorines [5]. Indeed, relatively elevated levels of the mouse homolog of ALDH-1, viz. AHD-2, account for the acquired resistance exhibited by two mouse lymphocytic leukemia sublines, viz. L1210/OAP and P388/CLA [1, 5–10]. Resistance was oxazaphosphorine-specific as would be expected since AHD-2 is not known to catalyze the detoxification of any of the other widely used antineoplastic agents nor of phosphoramide mustard, the oxazaphosphorine metabolite that effects the cytotoxic action of these agents.

* Descriptions of parts of this investigation have appeared in abstract form (Sreerama L and Sladek NE, *Proc Am Assoc Cancer Res* **32**: 352, 1991; Sreerama L and Sladek NE, *Proc Am Assoc Cancer Res* **33**: 447, 1992).

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‡ Abbreviations: ALDH, human aldehyde dehydrogenase; SSDH, succinic semialdehyde dehydrogenase; BADH, betaine aldehyde dehydrogenase; AHD, mouse aldehyde dehydrogenase; BSA, bovine serum albumin; PAGE, polyacrylamide gel eletrophoresis; SDS, sodium dodecyl sulfate; pI, isoelectric point; and mIU, milli-International Unit of enzyme activity (nmol NAD(P)H formed/min as used herein).

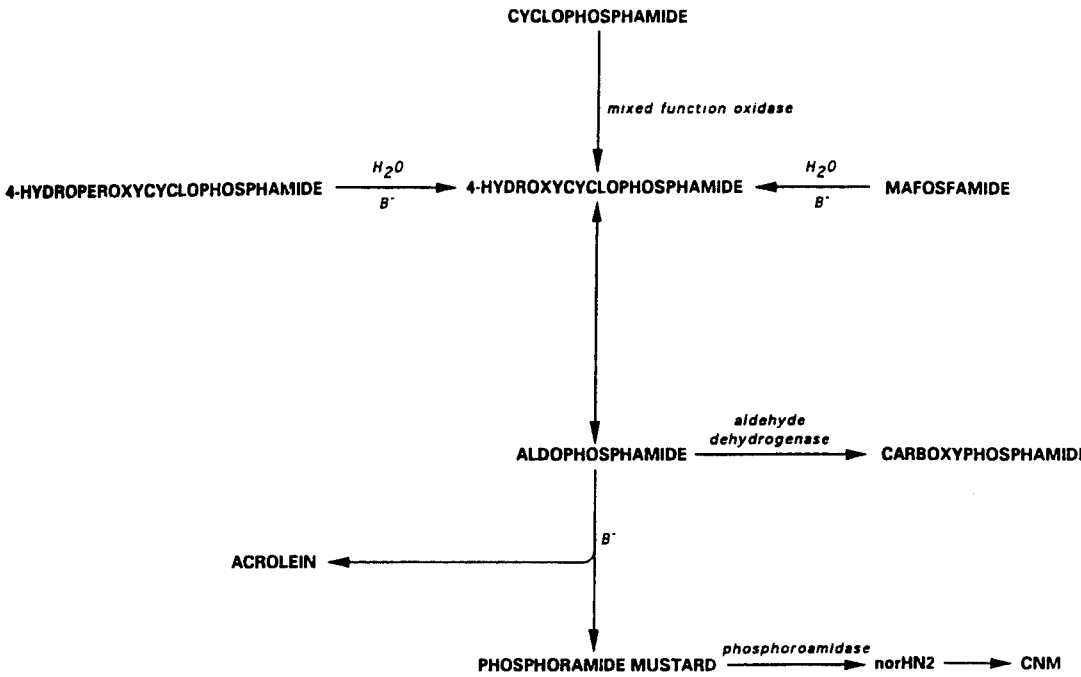

Fig. 1. Salient features of oxazaphosphorine metabolism. The prodrugs, cyclophosphamide, mafosfamide and 4-hydroperoxycyclophosphamide, each give rise to 4-hydroxycyclophosphamide which exists in equilibrium with its ring-opened tautomer, aldophosphamide. 4-Hydroxycyclophosphamide and aldophosphamide are, themselves, also without cytotoxic activity. However, aldophosphamide gives rise to acrolein and phosphoramide mustard, each of which is cytotoxic; the latter effects the bulk of the therapeutic action effected by the oxazaphosphorines [1]. Alternatively, aldophosphamide can be further oxidized to carboxyphosphamide by certain aldehyde dehydrogenases [1–3]. Carboxyphosphamide is without cytotoxic activity nor does it give rise to a cytotoxic metabolite. Aldehyde dehydrogenase-catalyzed oxidation of aldophosphamide to carboxyphosphamide is, therefore, properly viewed as an enzyme-catalyzed detoxification of the oxazaphosphorines. Key: norHN2, bis-(2-chloroethyl)-amine; and CNM, 3-(2-chloroethyl)-1,3-oxazolidine-2-one.

Frei and associates [11] have developed a human breast adenocarcinoma subline (termed MCF-7/OAP herein) that also exhibits oxazaphosphorine-specific acquired resistance. The expectation was that overexpression of, most probably, ALDH-1 or, alternatively, one of the other human aldehyde dehydrogenases known to catalyze the oxidation of aldophosphamide, viz. ALDH-2 or SSDH, would account for the oxazaphosphorine-specific resistance exhibited by these cells. Early on it became apparent that this expectation would not be realized. Instead, markedly elevated levels of a cytosolic class 3 enzyme that appeared to be similar, but not identical, to human stomach mucosa ALDH-3 were found. The investigations reported herein describe the isolation, physical and kinetic characterization, and identification of this enzyme.

MATERIALS AND METHODS

Materials. Mafosfamide and 4-hydroperoxycyclophosphamide were provided by Dr. J. Pöhl, Asta-Werke AG, Bielefeld, Germany. Phosphoramide mustard·cyclohexylamine was supplied by the Drug Development Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD. 4-Hydroperoxyifosfamide was prepared for us from ifosfamide by Dr. Kathleen Getman, University of Minnesota, according to the protocol described by Peter *et al.* [12]. Melphalan-HCl was supplied by Dr. G. M. Lyon, Jr., Burroughs Wellcome & Co., Research Triangle Park, NC. Actinomycin D was purchased from Calbiochem, Los Angeles, CA. Benzaldehyde, 4-pyridinecarboxaldehyde, octanal, propionaldehyde, acetaldehyde, acrolein (99+%), bis-(2-chloroethyl)-amine and methyl sulfide (99+%) were purchased from the Aldrich Chemical Co., Milwaukee, WI. NAD, NADP, NADH, NADPH, glutathione (reduced form), pyrazole, betaine aldehyde, succinic semialdehyde, γ-aminobutyraldehyde diethyl acetal (90%), DL-Δ^1-pyrroline-5-carboxylate-2,4-dinitrophenylhydrazone-HCl, glyceraldehyde 3-phosphate diethyl acetal monobarium salt, glyceraldehyde 3-phosphate dehydrogenase, bovine serum albumin (BSA; Fraction V), *p*-nitrophenyl acetate, Lubrol®, L-glutamine, Coomassie Brilliant Blue R-250, nitroblue tetrazolium, phenazine methosulfate, 2-(*N*-morpholino)ethane sulfonic acid, dithiothreitol, Freund's complete adjuvant, Freund's incomplete adjuvant, 1-chloro-2,4-dinitrobenzene, *p*-chloromercuribenzoic acid, disulfiram, menadione, ethacrynic acid, Reactive Blue 2-Sepharose CL 6B, Sephacryl S-200, standard protein marker kits for gel permeation, sodium dodecyl sulfate–

polyacrylamide gel electrophoresis (SDS–PAGE), gradient PAGE, and anti-chicken IgG alkaline phosphatase conjugate were purchased from the Sigma Chemical Co., St. Louis, MO. Fetal bovine serum was obtained from Hyclone Laboratories, Logan, UT. Ultrafiltration membranes (YM-30) were purchased from Amicon Division, W. R. Grace & Co., Danvers, MA. DEAE-Sephacel, CM-Sepharose CL 6B, PD-10 (Sephadex G-25) columns, Ampholine PAGplates® (pH 3.5–9.5) and an isoelectrofocusing marker kit were products of Pharmacia-LKB Biotechnology, Piscataway, NJ. Protein assay dye reagent concentrate, acrylamide and *N,N'*-methylene-bis-acrylamide were obtained from Bio-Rad Laboratories, Richmond, CA. Immobilon-PVDF transfer membrane was purchased from the Millipore Corp., Bedford, MA. Gentamicin (50 mg/mL), trypsin 2.5% (lyophilized powder; 10X) and Dulbecco's modified Eagle medium (powder; low glucose) were purchased from Gibco Laboratories, Grand Island, NY. Chloral hydrate and doxorubicin-HCl were purchased from the University of Minnesota Hospital Pharmacy, Minneapolis, MN. All other chemicals and reagents were of analytical grade.

Aldophosphamide was generated in aqueous solution by chemical reduction of 4-hydroperoxycyclophosphamide using methyl sulfide (99+%) as the reducing agent [3]. Glutamic-γ-semialdehyde was prepared from DL-Δ^1-pyrroline-5-carboxylate-2,4-dinitrophenylhydrazone-HCl according to the method of Mezl and Knox [13]. γ-Aminobutyraldehyde was prepared from γ-aminobutyraldehyde diethyl acetal by acid hydrolysis according to the method of Ambroziak and Pietruszko [14]. Glyceraldehyde 3-phosphate was prepared by acid hydrolysis of glyceraldehyde 3-phosphate diethyl acetal monobarium salt, and its concentration was determined using glyceraldehyde 3-phosphate dehydrogenase according to the protocol provided by the manufacturer. Homogenization medium was 1.15% (w/v) KCl and 1 mM EDTA in aqueous solution, pH 7.4. Drug-exposure medium was fetal bovine serum (10%) in phosphate-buffered saline-based solution, pH 7.4, prepared as previously described [8]. Growth medium was fetal bovine serum (10%) in Dulbecco's modified Eagle medium supplemented with L-glutamine (2 mM), sodium bicarbonate (3.7 g/L) and gentamicin (50 mg/L). Buffer A was 25 mM 2-(*N*-morpholino)ethane sulfonic acid, pH 6.5, supplemented with 1 mM EDTA and 0.05% dithiothreitol. Buffer B was 25 mM sodium phosphate, pH 7.5, supplemented with 1 mM EDTA and 0.05% dithiothreitol. Buffer C was 25 mM Tris–HCl in normal saline, pH 7.5.

Human MCF-7 breast adenocarcinoma cells sensitive (MCF-7/0) and resistant (MCF-7/OAP) to oxazaphosphorines, and growing in monolayer culture, were obtained originally from Dr. B. Teicher, Dana-Farber Cancer Institute, Boston, MA. They were propagated at 37° in dishes/flasks containing growth medium; the atmosphere of 5% CO_2 in air was fully humidified. Mean population-doubling times were approximately 25 (MCF-7/0) and 30 (MCF-7/OAP) hr. Mean plating efficiencies were approximately 50% in each case.

Cultured tumor cells in asynchronous exponential growth were submitted to trypsinization (0.25%) and then harvested by low-speed centrifugation (500 *g* for 10 min). After washing once with drug-exposure medium, they were resuspended in drug-exposure medium and checked for viability (usually greater than 95% as judged by trypan blue exclusion; preparations exhibiting less than 85% viability were discarded). This was the preparation used in the colony-forming assays. Cells were further handled in several different ways when enzyme activity in cell-free fractions was to be quantified or when enzymes were to be extracted and purified. Usually the cells were again harvested by low speed centrifugation, resuspended (1×10^7 cells/mL) in homogenization medium, and then used. Infrequently, this suspension was stored at −20° until used. Sometimes the harvested cells were stored at −20° as a pellet overlaid with homogenization medium until used. Enzyme activity was essentially unaffected by freezing and storage at −20°.

Except for liver, all human tissues were obtained from the Tissue Procurement Facility, University of Alabama Comprehensive Cancer Center, Birmingham, AL, through the Cooperative Human Tissue Network, Midwestern Division, Columbus, OH. Liver was obtained through the Liver Procurement and Distribution System, University of Minnesota, Minneapolis, MN. The suppliers certified all samples to be nonpathological. Breast samples were from a 19-, a 51-, a 59- and a 61-year-old Caucasian female; each was frozen at −70° within 3–5 hr of surgical removal and was stored at this temperature until used. Stomach, lung and kidney were from a 47-year-old Caucasian male who died of severe coronary heart disease, a 16-year-old Caucasian male who died of injuries sustained in a motor vehicle accident, and a 4½-month-old Caucasian female who died of sudden infant death syndrome, respectively. Each was surgically removed 4–6 hr post-mortem and was immediately frozen and stored at −70° until used. Liver was from a 22-year-old Caucasian male who died of injuries sustained in a motor vehicle accident. It was kept at 0–4° and delivered to us within 12 hr of donation; upon delivery it was frozen and stored at −70° until used. Placenta was kept at 0–4° and delivered to us within 24 hr of donation; it was assayed immediately for enzyme activity, i.e. without ever being frozen.

Purified ALDH-1, ALDH-2 and SSDH [3] were provided by Dr. P. A. Dockham.

Drug exposure and colony-forming assay. Freshly harvested cells were diluted with drug-exposure medium to a concentration of 1×10^5 cells/mL and were exposed to various drugs or the appropriate vehicle for 30 min at pH 7.4 and 37° in air. Except for ethacrynic acid, 1-chloro-2,4-dinitrobenzene, actinomycin D and menadione, all of the test drugs were dissolved in double-deionized water. Ethacrynic acid and 1-chloro-2,4-dinitrobenzene were dissolved in 50% ethanol. Actinomycin D and menadione were dissolved in absolute ethanol. All of the drug solutions were sterilized by passage through 0.22 μm Millipore filters; all were used within 1 hr of preparation and were kept at approximately 4° prior to their use. Drug solutions were added to tumor

cell suspensions in a volume of 0.1 mL; the final volume of the tumor cell suspension was 5 mL. Ethanol, at the concentrations used, was not toxic to tumor cells. At the end of the 30-min incubation periods, the cells were placed in an ice-bath and allowed to chill for 5 min. They were then harvested by low-speed centrifugation, washed with drug-free growth medium, and resuspended in growth medium at concentrations that allowed the transfer, in triplicate, of 10,000, 1000 and 100 cells, each in a volume of 1 mL, to 60 × 15 mm petri dishes containing 4 mL of growth medium. The cells were allowed to grow at 37° in a fully humidified 5% CO_2 in air atmosphere for 15 days after which time the medium was poured off, cells were stained with methylene blue dye, and colonies (≥50 cells) were counted.

Preparation of Lubrol®-treated whole homogenates. MCF-7/0 and MCF-7/OAP cells suspended in homogenization medium were lysed in an ice-bath by submitting them to sonication (Artek Dismembrator model 300; setting of 30) for a total period of 10 sec (divided into 3 bursts). The homogenate was then adjusted to 0.3% Lubrol®, vortexed, and centrifuged at 105,000 *g* and 4° for 1 hr. The resultant supernatant fraction was used for enzyme activity assay.

Lubrol®-treated 10% (w/v) liver whole homogenates were prepared as previously described [3]. Kidney Lubrol®-treated 10% (w/v) whole homogenates were prepared in an identical manner except that the homogenizing medium was Buffer B devoid of dithiothreitol. These preparations were used when enzyme activity was to be determined.

All preparations were transferred into Buffer B with the aid of Pharmacia PD-10 columns when electrophoresis was to be effected.

Preparation of subcellular fractions. Subcellular fractions of MCF-7/0 and MCF-7/OAP cells were prepared essentially as described before [15] except that 0.3% Lubrol®, rather than 0.3% deoxycholate, was used to solubilize the particulate fraction. Briefly, a Dounce homogenizer was used to homogenize cells in ice-cold homogenization medium and the homogenate was centrifuged at 105,000 *g* and 4° for 1 hr. The resultant supernatant (soluble) fraction was saved for enzyme activity assay; it was transferred into Buffer B or Buffer A with the aid of Pharmacia PD-10 columns when electrophoresis or chromatography, respectively, was to be effected. The pellet was washed once with homogenization medium and resuspended in homogenization medium containing 0.3% Lubrol®. This preparation was centrifuged as above and the resultant supernatant (solubilized particulate) fraction was saved for enzyme activity assay; it was transferred into Buffer B as above when electrophoresis was to be effected.

Soluble (105,000 *g* supernatant) fractions of lung, placenta and stomach mucosa (scraped away from the serosa with a scalpel) were prepared by homogenizing these tissues in Buffer B devoid of dithiothreitol, and submitting the 10% (w/v) homogenates to centrifugation at 105,000 *g* and 4° for 1 hr. The soluble (105,000 *g* supernatant) fraction of the breast tissue was prepared in an identical manner except that this tissue was first submitted to disruption in a Waring blender for 1 min before it [50% (w/v) homogenate] was submitted to further homogenization in a Dounce homogenizer. These preparations were used when enzyme activity was to be determined. All of the preparations were transferred into Buffer B with the aid of Pharmacia PD-10 columns when electrophoresis was to be effected or when the preparation, viz. stomach mucosa soluble fraction, was to undergo chromatography.

Enzyme assays. Aldehyde dehydrogenase activity was quantified spectrophotometrically, essentially as previously described [2, 3]. The reaction mixture (1 mL, pH 8.1) contained substrate (aldehyde), NAD (1 or 4 mM) or NADP (4 mM), pyrazole (0.1 mM), glutathione (5 mM), EDTA (1 mM), tetrasodium pyrophosphate (32 mM), crude fraction or (semi)purified aldehyde dehydrogenase, and, in some experiments, a potential inhibitor/modulator of the enzyme activity. Some of the substrates/inhibitors/modulators, viz. octanal, propionaldehyde and disulfiram, were dissolved in methanol and added to the reaction mixture in a volume not exceeding 50 μL. Aldehyde dehydrogenase activity was unaffected by this amount of methanol in the reaction mixture. The reaction was initiated by the addition of aldehyde and was followed at 37° by monitoring the appearance of NAD(P)H at 340 nm in a Beckman DU-70 automated recording spectrophotometer. All rates were determined in duplicate.

Esterase activity was determined by monitoring the increase in absorbance at 400 nm due to *p*-nitrophenol production from *p*-nitrophenyl acetate [16].

Protein determination. Protein concentrations were estimated by the method of Bradford [17], using commercially available Bio-Rad protein assay regaent and BSA as the standard.

Chromatographic purification of aldehyde dehydrogenases. DEAE-Sephacel, CM-Sepharose CL 6B and Reactive Blue 2-Sepharose CL 6B column chromatography was performed at 4–6°. All buffers were degassed prior to use. Linear flow rates were 30, 30 and 20 mL/hr, respectively. Concentration of samples was with an Amicon Diaflo concentrator fitted with a YM-30 membrane and pressurized with nitrogen. Protein concentrations of samples loaded onto columns never exceeded 15 mg/mL and typically were much less. Benzaldehyde (4 mM) and NAD (1 mM) were used as the substrate and cofactor, respectively, to monitor aldehyde dehydrogenase activity in column eluates. Protein was monitored at 280 nm with an ISCO UA-5 absorbance monitor.

Analytical and preparative non-denaturing PAGE. Non-denaturing PAGE was carried out essentially as described by Davis [18] to monitor enzyme purification. A discontinuous gel system, 8% separating gel (pH 8.8) and 4% stacking gel (pH 6.8), was used for this purpose. Slab gels were 70 × 80 × 0.5 mm. Samples (1 mg protein/mL) were prepared in an aqueous solution of 0.5 M Tris–HCl, pH 6.8, containing 10% glycerol or 20% sucrose, and 2 μL 0.05% bromophenol blue. Aliquots (25 μL) were electrophoresed at 4° with the aid of a Bio-Rad Protein-II (mini) vertical slab gel electrophoretic

system by applying a constant voltage (200 V) until the tracking dye reached the lower tip of the gel.

Preparative PAGE was performed as above except that the gels were 200 × 160 × 1.5 mm in size and electrophoresis was performed in a Bio-Rad Protein-II system at low constant voltage (75 V).

Isoelectric focusing. Isoelectric focusing was as described by Manthey *et al.* [2], except that commercially available Ampholine PAGplates® (250 × 115 × 1 mm) containing 10% glycerol and 2% ampholite (pH 3.5 to 9.5), and a constant power output of 20 W for 3000 V-hr, were used for this purpose. Samples (50 μL) were loaded onto the gel surface with the aid of sample applicators and electrophoresis was effected at 4–6°. A broad range standard protein isoelectric point (pI) marker kit was used to assign the pI values to aldehyde dehydrogenases.

Gels were stained for both aldehyde dehydrogenase activity and protein. They were immersed in an aqueous solution containing 32 mM sodium pyrophosphate, pH 8.1, 1 mM nitroblue tetrazolium, 130 μM phenazine methosulfate, 1 mM pyrazole, 4 mM NAD(P) and aldehyde substrate for 20–45 min at 37° to visualize aldehyde dehydrogenases. Staining solutions lacking either NAD(P) or aldehyde were used to develop control gels, i.e. to distinguish the enzymes of interest from other enzymes that directly or indirectly transfer electrons to nitroblue tetrazolium when aldehyde or NAD(P) alone is present. Coomassie Brilliant Blue R-250 (0.05%) was used to visualize proteins. The gels were destained by slow leaching in a destaining solution containing water:methanol:acetic acid (6:3:1).

Molecular weight determinations. Native molecular weights of the purified enzymes were determined by gel filtration on a Sephacryl S-200 column (1 × 50 cm) equilibrated with Buffer B. They were also determined by linear gradient gel electrophoresis as described by Margolis and Kenrick [19].

Subunit molecular weights of the two purified aldehyde dehydrogenases were determined on SDS–polyacrylamide gels (12%) essentially according to the method of Laemmli [20].

Preparation of antibodies. Antibodies against stomach mucosa ALDH-3 were obtained by immunization of egg-laying hens (White Leghorn) essentially according to the method of Gassmann *et al.* [21]. Briefly, stomach mucosa ALDH-3 (350 μg in 0.5 mL of a phosphate-buffered saline solution, pH 7.4) was mixed with an equal volume of Freund's complete adjuvant and emulsified. One-half of the emulsion was then injected subcutaneously into the pectoral muscle (two sites) of each of two birds. A booster injection (100 μg of enzyme in phosphate-buffered saline solution, pH 7.4, and Freund's incomplete adjuvant) was given 3 weeks later. Eggs were collected daily, marked and stored at 4° until used. Antibodies (IgY) were isolated from egg yolk by polyethylene glycol precipitation and were partially purified by DEAE-Sephacel chromatography. As judged by ELISA (1 μg purified enzyme) and by SDS–PAGE/immunoblot assay (5 μg purified enzyme), anti-stomach mucosa ALDH-3 IgY prepared in this manner was not cross-reactive with ALDH-1 or ALDH-2.

Immunoblot analysis. Purified stomach mucosa and MCF-7/OAP ALDH-3s were electrophoresed on a 12% SDS–polyacrylamide gel and electrotransferred onto Immobilon-PVDF transfer membrane using a Bio-Rad semidry blotter [22]. The transfer membrane was processed at room temperature by, first, shaking it for 2 hr in a blocking solution of 5% (w/v) instant nonfat milk in Buffer C; second, shaking it for 2 hr with partially purified anti-stomach ALDH-3 IgY suspended (1:500) in the blocking solution; third, vigorously shaking it three times, 10 min each time, in Buffer C; fourth, incubating it for 2 hr with a secondary antibody (anti-chicken IgG, 1:1000) coupled to alkaline phosphatase; fifth, vigorously shaking it three times, 10 min each time, in Buffer C; and lastly, staining it for alkaline phosphatase activity.

Purified stomach mucosa and MCF-7/OAP ALDH-3s were also electrophoresed on Ampholine PAGplates® and electrotransferred onto transfer membranes essentially as described by Dunn [23]. Thus, the gel was removed from the backing, rinsed with an aqueous solution, pH 9.9, containing 10 mM $NaHCO_3$ and 3 mM $NaCO_3$ but devoid of methanol, and electrotransferred with the same solution and a Bio-Rad semidry blotter. Further processing of the membrane was as described above except that the blocking solution was 5% BSA (w/v), rather than 5% instant nonfat milk, in Buffer C.

Data analysis. Double-reciprocal plots of initial rates versus substrate concentrations were used to estimate all K_m and V_{max} values. Initial rates were determined in duplicate with each of five to twelve substrate concentrations to generate each value.

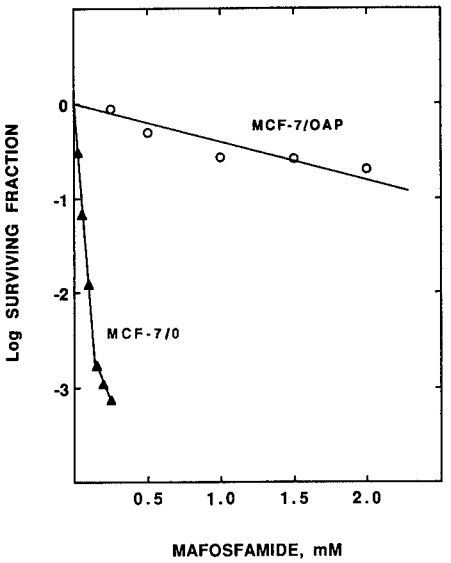

Fig. 2. Sensitivity of cultured MCF-7/0 and MCF-7/OAP cells to mafosfamide. Cultured oxazaphosphorine-sensitive MCF-7/0 (▲), and oxazaphosphorine-resistant MCF-7/OAP (○), cells were incubated with mafosfamide for 30 min at 37°. The cells were then harvested and grown in a drug-free medium. The colony-forming assay described in Materials and Methods was used to determine surviving fractions. Each point is the mean of measurements on triplicate cultures.

Table 1. Sensitivity of MCF-7/0 and MCF-7/OAP cells to mafosfamide and other cytotoxic agents*

Drug	Exposure time (min)	LC_{90} (μM)		Ratio
		MCF-7/0	MCF-7/OAP	
Mafosfamide	30	50	>2,000	>40
4-Hydroperoxyifosfamide	30	90	>1,000	>11
Phosphoramide mustard	30	800	1,530	1.9
Acrolein	30	34	50	1.5
Bis-(2-chloroethyl)-amine	30	134	420	3.1
Melphalan	30	4	5	1.3
Doxorubicin	30	1.5	2.2	1.5
Actinomycin D	30	1.0	1.7	1.7
Menadione	30	32	30	1.0
1-Chloro-2,4-dinitrobenzene	35	12	12	1.0
Ethacrynic acid	35	312	378	1.2

* Cultured oxazaphosphorine-sensitive MCF-7/0, or oxazaphosphorine-resistant MCF-7/OAP, cells were exposed at 37° to five to seven concentrations of each of the cytotoxic agents for the time periods indicated. The cells were then harvested and grown in a drug-free medium. The colony-forming assay described in Materials and Methods was used to determine surviving fractions. The resultant data i.e. the means of measurements on triplicate cultures at each concentration, were plotted as illustrated in Fig. 2. The LC_{90} (concentration of drug required to effect a 90% cell kill) values were obtained from such plots.

Wilkinson weighted linear regression analysis [24] was used to fit lines to the double-reciprocal plot values.

Computer-assisted unweighted regression analysis was carried out using the STATView® (Brainpower, Inc., Calabas, CA) statistical program to generate all other straight line functions.

RESULTS

Frei and associates [11] developed a subline of the human breast adenocarcinoma MCF-7/0 cell line, viz. MCF-7/OAP, that is relatively insensitive to 4-hydroperoxycyclophosphamide by growing the parent line in the presence of increasing amounts of 4-hydroperoxycyclophosphamide over a period of several months. The subline had not lost sensitivity to several other agents, viz. mechlorethamine, melphalan, thioTEPA, mitomycin C, *N,N'*-bis(2-chloroethyl)-*N*-nitrosourea (BCNU) and cisplatin, that are chemically and/or pharmacologically related but that are not oxazaphosphorines. In our hands, MCF-7/OAP cells exhibited a cross-resistance to two other oxazaphosphorines, viz. mafosfamide and 4-hydroperoxyifosfamide, but not to any of several oxazaphosphorine metabolites, including phosphoramide mustard, that are not oxazaphosphorines, or to several other agents, some of which are used as antineoplastic agents, but none of which are oxazaphosphorines (Fig. 2 and Table 1). Thus, MCF-7/OAP cells exhibit an oxazaphosphorine-specific acquired resistance.

Table 2. Aldehyde dehydrogenase activity in the soluble (105,000 *g* supernatant) fractions of MCF-7/0 and MCF-7/OAP cells*

Substrate (concentration)	Cofactor (4 mM)	Aldehyde dehydrogenase activity (mIU/10^7 cells)	
		MCF-7/0	MCF-7/OAP
Aldophosphamide	NAD	0†	2.8 ± 0.6
(160 μM)	NADP	0	0
Acetaldehyde	NAD	1.4	6.6
(4 mM)	NADP	0	0
Benzaldehyde	NAD	1.7 ± 0.3	110 ± 6.6
(4 mM)	NADP	1.9 ± 0.3	254 ± 15.8

* Soluble fractions were freshly prepared from tumor cells in exponential growth, and aldehyde dehydrogenase activity in such fractions obtained from 2×10^5 to 1×10^7 cells was determined as described in Materials and Methods. Each value is the mean of duplicate determinations on each of one to three samples. Standard errors of these values are presented in those cases where determinations were on three samples.

† Subsequently, it will be shown that the MCF-7/0 cell line does contain very small amounts (below detectable levels in the present experiment) of an aldehyde dehydrogenase, seemingly Type-2 ALDH-3, that catalyzes the NAD-dependent oxidation of aldophosphamide to carboxyphosphamide.

The expectation was that NAD(P)-dependent enzyme-catalyzed detoxification of the oxazaphosphorines would be elevated markedly in the MCF-7/OAP cells, thereby accounting for the oxazaphosphorine-specific acquired resistance. NAD(P)-dependent enzyme-catalyzed oxidation of aldophosphamide to carboxyphosphamide was elevated in the resistant subline (Table 2), but the activity was of such a small magnitude that any notion that increased oxidation of aldophosphamide to carboxyphosphamide accounts for oxazaphosphorine-specific acquired resistance is highly problematical. Enzyme-catalyzed oxidation of acetaldehyde was also minimal in these cells, but that of benzaldehyde was elevated markedly and of a large magnitude regardless of whether NAD or NADP was used as the cofactor. Pyridine nucleotide-dependent oxidation of glutamic-γ-semialdehyde, succinic semialdehyde, betaine aldehyde or glyceraldehyde 3-phosphate was not catalyzed by either subcellular (soluble and particulate) fractions or Lubrol®-solubilized whole homogenates of the two cell lines (data not presented). Aldehyde dehydrogenase-catalyzed oxidation of aldophosphamide, acetaldehyde and/or benzaldehyde, was confined to the cytosol, more accurately, the soluble (105,000 *g* supernatant) fraction in each cell line, i.e. it was not detected in particulate (105,000 *g* pellet) fractions (data not presented). Disulfiram (50 μM) and chloral hydrate (100 μM) did not inhibit the catalysis of aldehyde (acetaldehyde, benzaldehyde and octanal) oxidation by the soluble (105,000 *g* supernatant) fraction obtained from MCF-7/OAP cells (data not presented).

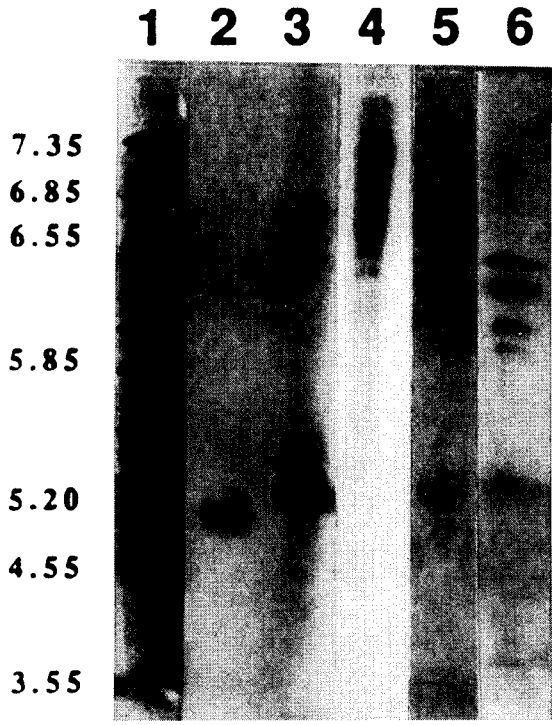

Fig. 3. Isoelectric focusing of human aldehyde dehydrogenases known to catalyze the oxidation of aldophosphamide to carboxyphosphamide, and whole homogenates of MCF-7/0 and MCF-7/OAP cells. Human aldehyde dehydrogenases known to catalyze the oxidation of aldophosphamide to carboxyphosphamide, viz. ALDH-2 (lane 2), ALDH-1 (lane 3) and SSDH (lane 4), Lubrol®-treated whole cell homogenates of MCF-7/0 (lane 5) and MCF-7/OAP (lane 6), and pI standards (lane 1) were subjected to isoelectric focusing as described in Materials and Methods. The amount of each purified enzyme, i.e. ALDH-1, ALDH-2 and SSDH, loaded onto the gel was sufficient to generate 1.5 to 2.0 nmol NADH/min as determined by spectrophotometric assay and the substrate with which the enzyme was ultimately stained. MCF-7/0 and MCF-7/OAP Lubrol®-treated whole homogenates loaded onto the gel were from 1×10^7 and 2.5×10^5 cells, respectively. The nitroblue tetrazolium-coupled enzyme activity stain described in Materials and Methods was used to visualize aldehyde dehydrogenases in lanes 2–6. Substrates were acetaldehyde (4 mM) for ALDH-1 and ALDH-2, succinic semialdehyde (100 μM) for SSDH, and benzaldehyde (4 mM) for aldehyde dehydrogenases present in tumor cell whole homogenates; the cofactor was NAD (4 mM). Coomassie Brilliant Blue R-250 was used to stain the pI standards. The relatively dark background in lane 5 is because MCF-7/0 cells contain comparatively very little of the enzyme necessitating that this part of the gel be left in the staining solution for a relatively much longer time period in order to visualize the enzyme. In a separate experiment (not shown) ALDH-1 or ALDH-2 activity was also not detectable in Lubrol®-treated whole homogenates of MCF-7/0 and MCF-7/OAP cells when acetaldehyde (4 mM) was used as the substrate to assay for such activity.

ALDH-3 is a cytosolic class 3 aldehyde dehydrogenase that utilizes both NAD and NADP as cofactors, prefers aromatic and long-chain aliphatic aldehydes to short-chain aliphatic aldehydes as substrates, and is relatively insensitive to disulfiram inhibition [4]. Moreover, pI values (*ca.* 5.7 to 6.4) for this enzyme are substantially different from other known aldehyde dehydrogenases. It is found in several, but not all, human organs/tissues/cells [4, 25], and an enzyme with physical and catalytic properties similar to the human enzyme has been found in various organs/tissues/cells of a number of mammalian species [25].

Electrofocusing experiments (Fig. 3) revealed that an aldehyde dehydrogenase exhibiting pI values and an electrofocusing pattern very similar, if not identical, to those reported for ALDH-3 was present in both MCF-7/0 and MCF-7/OAP cells, that the amount of this enzyme was substantially greater in the MCF-7/OAP cell line, and that detectable amounts of other aldehyde dehydrogenases, including those known to catalyze the oxidation of aldophosphamide, viz. ALDH-1, ALDH-2 and SSDH, were not present in either cell line.

The only well-characterized human cytosolic ALDH-3 is the one constitutively present in stomach mucosa [26, 27]. However, although limited, available data indicate that the ALDH-3s identified thus far in other human tissues are identical to the stomach mucosa enzyme [4]. Side-by-side electrophoresis of the crude fractions prepared from stomach mucosa and MCF-7/OAP cells revealed that, while the MCF-7/OAP enzyme isoelectric-focused in a manner very similar to stomach mucosa ALDH-3, it did not do so in an identical manner (Fig. 4) suggesting that, whereas the MCF-7/OAP enzyme was indeed a cytosolic class 3 aldehyde dehydrogenase, it was different from the ALDH-3

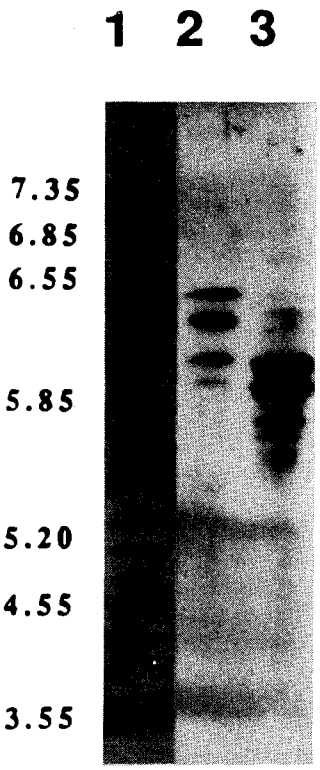

Fig. 4. Isoelectric focusing of aldehyde dehydrogenases present in the soluble (105,000 *g* supernatant) fractions of human stomach mucosa and Lubrol®-treated whole homogenates of MCF-7/OAP cells. An aliquot of a human stomach mucosa soluble fraction sufficient to generate 5.0 nmol NADH/min when benzaldehyde was the substrate, a Lubrol®-treated whole homogenate obtained from 2.5×10^5 MCF-7/OAP cells, and pI standards were loaded onto the gel and electrofocused as described in Materials and Methods. Lane 1 (pI standards) was stained for protein using Coomassie Brilliant Blue R-250. Lanes 2 (MCF-7/OAP whole cell homogenate) and 3 (human stomach mucosa soluble fraction) were stained for aldehyde dehydrogenase activity as described in Materials and Methods; benzaldehyde (4 mM) was the substrate and NAD (4 mM) was the cofactor.

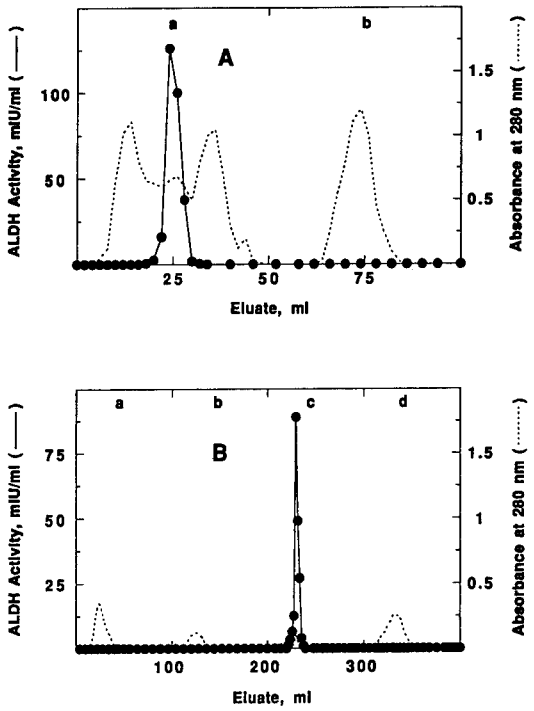

Fig. 5. Chromatographic purification of a class 3 aldehyde dehydrogenase present in MCF-7/OAP cells. (A) A concentrated (1.5 mL) soluble (105,000 *g* supernatant) fraction of MCF-7/OAP cells (5×10^7) was prepared as described in Materials and Methods and was then loaded onto a CM-Sepharose CL 6B column (1.5×20 cm) equilibrated with Buffer A. Elution was with (a) 50 mL Buffer A, followed by (b) 50 mL Buffer A supplemented with 700 mM NaCl. (B) CM-Sepharose CL 6B column eluates exhibiting aldehyde dehydrogenase activity were pooled (15 mL), concentrated (1 mL) as described in Materials and Methods, and loaded onto a Reactive Blue 2-Sepharose CL 6B column (1.5×20 cm) equilibrated with Buffer A. The loaded column was successively eluted with (a) 100 mL Buffer A, (b) 100 mL Buffer B, (c) 100 mL Buffer B supplemented with 5 mM NAD, and (d) 100 mL Buffer B supplemented with 700 mM NaCl. Eluates were collected in 2-mL fractions. Benzaldehyde (4 mM) and NAD (1 mM) were used to monitor aldehyde dehydrogenase activity. Enzyme activities, yields, and fold-purifications are tabulated in Table 3.

Table 3. Purification of a cytosolic class 3 aldehyde dehydrogenase from MCF-7/OAP cells*

Purification step	Total activity† (mIU)	Yield (%)	Total protein (mg)	Specific activity (mIU/mg)	Fold-purification
Soluble (105,000 *g* supernatant) fraction	600	100	23.7	25	1
CM-Sepharose CL 6B chromatography	560	93	1.8	311	12
Reactive Blue 2-Sepharose CL 6B affinity chromatography	364	61	0.027	13,481	539
Preparative PAGE/ Electroelution	250	42	0.015	16,667	667

* Purification was as described in Fig. 5 and the text.

† Benzaldehyde (4 mM) and NAD (1 mM) were used as substrate and cofactor, respectively, to quantify aldehyde dehydrogenase activity.

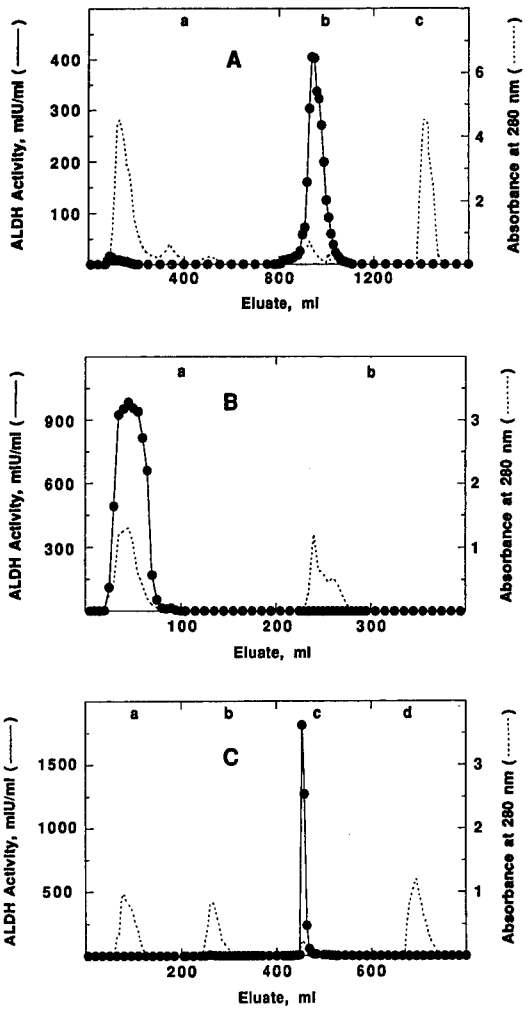

Fig. 6. Chromatographic purification of a class 3 aldehyde dehydrogenase present in human stomach mucosa. (A) A (50 mL) soluble (105,000 *g* supernatant) fraction of human stomach mucosa (4 g) was prepared as described in Materials and Methods and was then loaded onto a DEAE-Sephacel column (2.5 × 25 cm) equilibrated with Buffer B. The loaded column was successively eluted with (a) 750 mL Buffer B, (b) 400 mL Buffer B supplemented with 100 mM NaCl, and (c) 400 mL Buffer B supplemented with 700 mM NaCl. (B) DEAE-Sephacel column eluates exhibiting aldehyde dehydrogenase activity were pooled (150 mL), concentrated (5 mL) as described in Materials and Methods, transferred into Buffer A with the aid of Pharmacia PD-10 columns, and loaded onto a CM-Sepharose CL 6B column (1.5 × 35 cm) equilibrated with Buffer A. Elution was with (a) 195 mL Buffer A, followed by (b) 200 mL Buffer A supplemented with 700 mM NaCl. (C) CM-Sepharose CL 6B column eluates exhibiting aldehyde dehydrogenase activity were pooled (65 mL), concentrated (5 mL) as described in Materials and Methods, and loaded onto a Reactive Blue 2-Sepharose CL 6B column (1.5 × 35 cm) equilibrated with Buffer A. The loaded column was successively eluted with (a) 195 mL Buffer A, (b) 200 mL Buffer B, (c) 200 mL Buffer B containing 5 mM NAD, and (d) 200 mL Buffer B supplemented with 700 mM NaCl. Eluates were collected in fractions of 5–10 mL. Benzaldehyde (4 mM) and NAD (1 mM) were used to monitor aldehyde dehydrogenase activity. Enzyme activities, yields, and fold-purifications are tabulated in Table 4.

constitutively present in human stomach and other tissues. This notion was pursued in the next series of experiments. Purified enzymes were used for this purpose.

Purification of the MCF-7/OAP enzyme was as shown in Fig. 5. Small amounts of nonspecific proteins were still present in the enzyme preparation after Reactive Blue 2-Sepharose CL 6B chromatography. Therefore, it was submitted to further fractionation on preparative PAGE. Specific activity of the final product was 16,667 mIU/mg protein (Table 3). Purification of the stomach mucosa enzyme was as shown in Fig. 6. Specific activity of the final product was 32,951 mIU/mg protein (Table 4). The specific activity exhibited by the final product obtained by Eckey *et al.* [27] was much lower; that obtained by Wang *et al.* [26] was very similar. As judged by isoelectric focusing, non-denaturing linear gradient PAGE, and SDS–PAGE (Figs. 7–9, respectively) each of the enzymes had been purified to homogeneity.

As judged by a number of criteria, the purified enzymes, while resembling each other, were not identical entities. Four bands of catalytic activity, two major and two minor, were observed when the MCF-7/OAP enzyme was subjected to isoelectric focusing (Fig. 7); pI values for the major and minor bands were 6.35 and 6.45, and 6.0 and 6.25, respectively. In contrast, five bands of catalytic activity, two major and three minor, were observed when the stomach mucosa enzyme was isoelectric-focused; pI values for the major and minor bands were 6.0 and 6.25, and 5.75, 5.85 and 6.35, respectively. As determined by non-denaturing linear gradient PAGE, the relative molecular mass of each of the native enzymes was 110 kDa (Fig. 8), but relative molecular masses of 125 and 108 kDa were obtained for the MCF-7/OAP and stomach mucosa enzymes, respectively, when Sephacryl S-200 gel permeation column chromatography was utilized to make these determinations (Fig. 10). Subunit relative molecular masses for the MCF-7/OAP and stomach mucosa enzyme were 40 and 54.5 kDa, respectively (Fig. 9). Anti-stomach mucosa ALDH-3 IgY recognized native, but not denatured, MCF-7/OAP ALDH-3 (Figs. 11 and 12, respectively). Least recognized of the native MCF-7/OAP enzyme were the two major bands (pI = 6.35 and 6.45). Failure to recognize the MCF-7/OAP enzyme subunit may be due to the loss of an epitopic recognition site on denaturation of the native enzyme. According to this scenario, the epitopic recognition site would also be lost by the stomach mucosa enzyme upon denaturation but some part of the primary structure of this enzyme would still be recognized by the antibody. Further, the primary structure of this part of the MCF-7/OAP enzyme subunit would differ to the extent that it would not be recognized by the antibody.

The pI and apparent molecular weight values reported herein for the native and denatured stomach mucosa enzyme are in close agreement with those reported by others [26, 27]. The latter suggest that the native enzyme is a dimer [4, 26, 27]. With the very large caveat that molecular weights estimated by gel permeation chromatography or linear

Table 4. Purification of a cytosolic class 3 aldehyde dehydrogenase from human stomach mucosa*

Purification step	Total activity† (mIU)	Yield (%)	Total protein (mg)	Specific activity (mIU/mg)	Fold-purification
Soluble (105,000 *g* supernatant) fraction	33,500	100	430	78	1
DEAE-Sephacel chromatography	28,475	85	26.1	1,092	14
CM-Sepharose CL 6B chromatography	25,125	75	16.3	1,542	20
Reactive Blue 2-Sepharose CL 6B affinity chromatography	20,100	60	0.61	32,951	422

* Purification was as described in Fig. 6 and the text.
† Benzaldehyde (4 mM) and NAD (1 mM) were used as substrate and cofactor, respectively, to quantify aldehyde dehydrogenase activity.

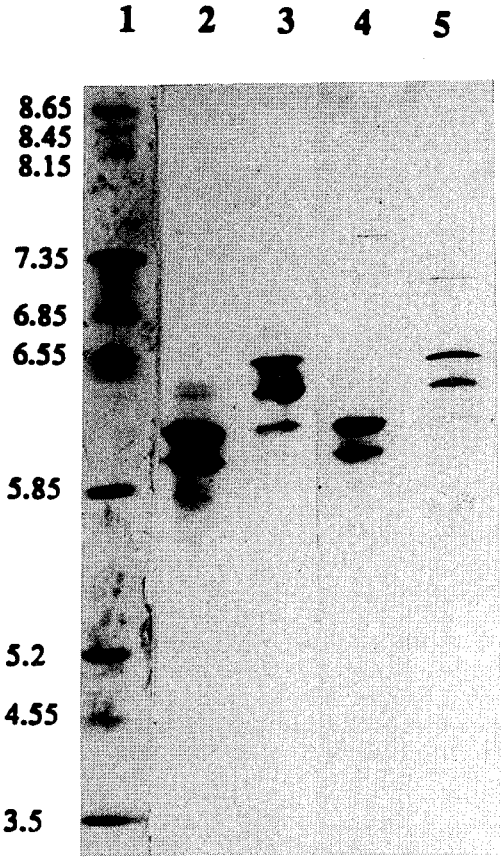

Fig. 7. Isoelectric focusing of purified stomach mucosa and MCF-7/OAP aldehyde dehydrogenases. Purified enzymes (10 μg each; lanes 2–5) and pI standards (lane 1) were electrofocused as described in Materials and Methods. Lanes 1 (pI standards), 4 (stomach mucosa aldehyde dehydrogenase) and 5 (MCF-7/OAP aldehyde dehydrogenase) were stained with Coomassie Brilliant Blue R-250 for the presence of proteins. Lanes 2 (stomach mucosa aldehyde dehydrogenase) and 3 (MCF-7/OAP aldehyde dehydrogenase) were stained for enzyme activity as described in Materials and Methods; benzaldehyde (4 mM) was the substrate and NAD (4 mM) was the cofactor.

gradient gel electrophoresis actually estimate relative molecular volumes (Stokes radii) and thus, for any given molecule, are accurate only to the extent that the molecular volume accurately reflects the molecular weight, the apparent molecular weight that we obtained for the MCF-7/OAP enzyme suggests that it may be a trimer when in its native form.

Temperature and pH optimums for catalytic activity are shown in Figs. 13 and 14, respectively. Each of the enzymes was optimally active at 37°. Energy of activation values, as determined by Arrhenius plots, were 15 and 14 kcals/mol for the MCF-7/OAP and stomach mucosa enzymes, respectively. Catalysis of benzaldehyde oxidation by the MCF-7/OAP enzyme (as well as that of aldophosphamide—data not shown) was optimum in the pH range approximately 8.0–8.5. Catalysis of benzaldehyde oxidation by the stomach mucosa enzyme was optimum over a slightly broader pH range, viz, approximately 8.0–9.0.

The MCF-7/OAP and stomach mucosa enzymes were each heat labile with the latter being slightly more so (Fig. 15). Crucial to the determination of meaningful kinetic constants, *vide infra*, full enzyme activity was retained by both enzymes for at least 10 min when they were exposed to a temperature of 37°. Retained enzyme activity as a function of storage (4°; 24 hr) pH is shown in Fig. 16. The MCF-7/OAP enzyme was optimally stable in the pH range 6.5–7.5. The stomach mucosa enzyme was optimally stable in the pH range 7.5–9.5.

The ability of the purified MCF-7/OAP enzyme to catalyze the oxidation of benzaldehyde was completely lost within 15 days when it was kept at 4° in Buffer B, pH 7.5, ±10% glycerol, whereas about 25% of the catalytic activity remained when the stomach mucosa enzyme was kept under identical conditions for 3 months (data not presented). Full catalytic activity was retained for at least 6 months by both enzymes when they were placed in Buffer B, pH 7.5, and stored frozen at −20° (data not presented). However, freezing and thawing of the MCF-7/OAP enzyme more than once caused a complete loss of catalytic activity whereas the stomach mucosa enzyme retained approximately

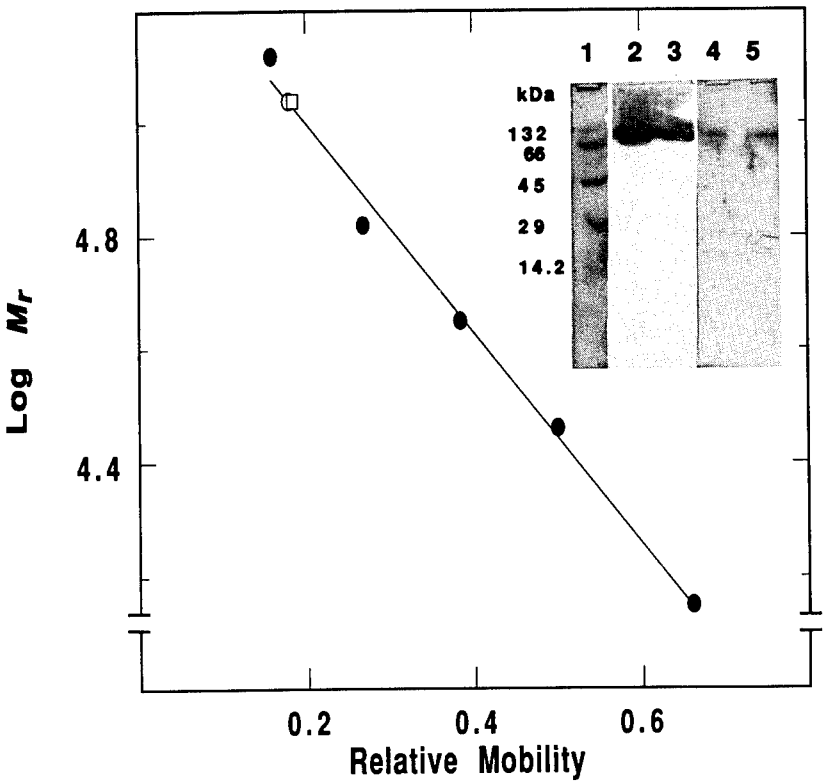

Fig. 8. Molecular weight determination of class 3 aldehyde dehydrogenases by non-denaturing linear gradient PAGE. Native molecular weights of the purified enzymes were determined as described in Materials and Methods using 5–20% non-denaturing linear gradient polyacrylamide gels. Molecular weight markers (●) were lactalbumin (14.2 kDa), carbonic anhydrase (29 kDa), ovalbumin (45 kDa), BSA monomer (66 kDa) and BSA dimer (132 kDa). Estimates of MCF-7/OAP (□) and stomach mucosa (○) aldehyde dehydrogenase molecular weights were made from the plot shown. Inset: Photograph of the original, developed gel. Lanes 1 (marker proteins), 4 (stomach mucosa aldehyde dehydrogenase) and 5 (MCF-7/OAP aldehyde dehydrogenase) were visualized for proteins by staining with Coomassie Brilliant Blue R-250. Lane 2 (stomach mucosa aldehyde dehydrogenase) and lane 3 (MCF-7/OAP aldehyde dehydrogenase) were stained for enzyme activity as described in Materials and Methods. Placed on gels were 10 μg of each purified protein.

Table 5. Kinetic properties of purified class 3 aldehyde dehydrogenases originally present in human MCF-7/OAP cells and stomach mucosa*

Substrate (mM)	Cofactor	MCF-7/OAP K_m (μM)	MCF-7/OAP V_{max} or v (mIU/mg)	MCF-7/OAP V_{max}/K_m (mIU/mg/μM)	Stomach mucosa K_m (μM)	Stomach mucosa V_{max} (mIU/mg)	Stomach mucosa V_{max}/K_m (mIU/mg/μM)
Benzaldehyde	NAD	640	16,900†	26	505	32,000	63
(0.1–4.0)	NADP	640	29,600†	46	486	51,190	105
4-Pyridinecarboxaldehyde	NAD	91	5,333†	59	190	21,250	112
(0.02–4.0)	NADP	91	8,886†	98	190	49,019	258
Octanal	NAD	67	7,207†	108	113	15,226	135
(0.01–0.5)	NADP	67	9,195†	137	104	19,545	188
Propionaldehyde	NAD	ND‡	545§		19,060	16,388	0.9
(4.0–60)	NADP	ND	573§		19,000	23,290	1.2
Acetaldehyde	NAD	ND	229§		80,000	20,500	0.3
(4.0–200)	NADP	ND	0§		81,000	26,660	0.3
Aldophosphamide	NAD	640	573†	0.9		0	
(0.16–1.12)	NADP		0†			0	

* Kinetic constants were determined as described in Materials and Methods. Stock purified enzyme preparations were in Buffer B and were added to the reaction mixture in a volume of 100 μL. NAD and NADP concentrations were 1.0 and 4.0 mM, respectively. Each value is the mean of three determinations.

† V_{max}.

‡ ND: not determined.

§ v; the substrate concentration was 4.0 mM.

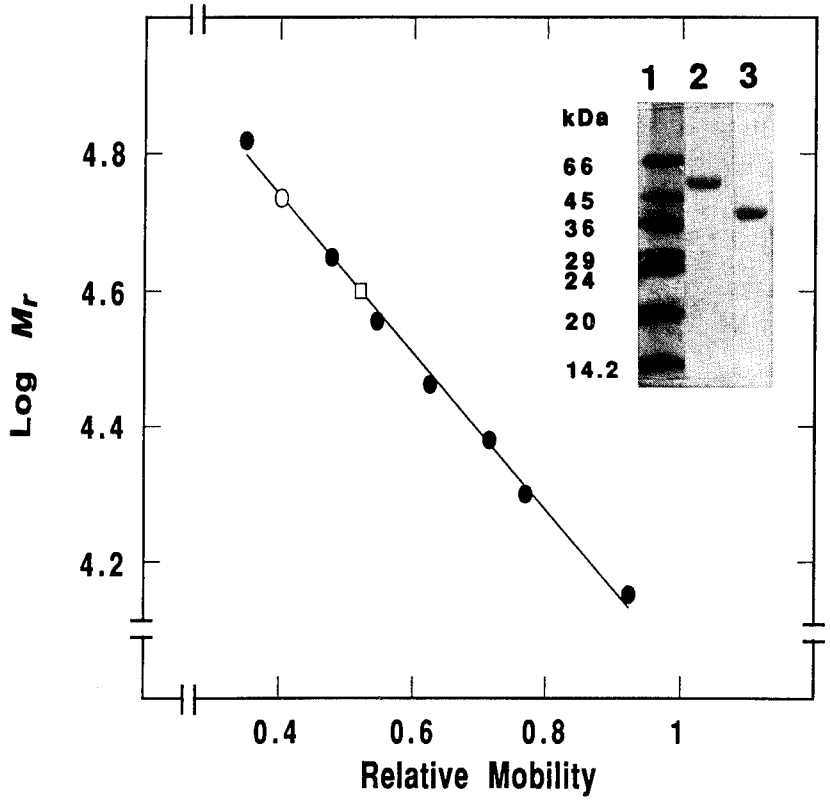

Fig. 9. Subunit molecular weight determination of class 3 aldehyde dehydrogenases by SDS–PAGE. Subunit molecular weights of the purified enzymes were determined as described in Materials and Methods. Molecular weight markers (●) were lactalbumin (14.2 kDa), trypsin inhibitor (20 kDa), trypsinogen (24 kDa), carbonic anhydrase (29 kDa), glyceraldehyde 3-phosphate dehydrogenase (36 kDa), ovalbumin (45 kDa) and BSA monomer (66 kDa). Estimates of MCF-7/OAP (□) and stomach mucosa (○) aldehyde dehydrogenase subunit molecular weights were made from the plot shown. Inset: Photograph of the original, developed gel. Proteins were visualized by staining with Coomassie Brilliant Blue R-250. Lane 1, marker proteins; lane 2, stomach mucosa aldehyde dehydrogenase (25 μg); and lane 3, MCF-7/OAP aldehyde dehydrogenase (25 μg).

40% of its catalytic activity even after being frozen and thawed eight times (data not presented). Regardless of the storage conditions and the amount of enzyme activity lost, new enzyme activity bands were not observed with either enzyme after isoelectric focusing (data not presented).

The MCF-7/OAP and stomach mucosa enzymes each catalyzed the oxidation of a variety of aliphatic and aromatic aldehydes (Table 5). In each case, short-chain aliphatic aldehydes were, as compared with long-chain aliphatic and aromatic aldehydes, poor substrates. The stomach enzyme did not catalyze the oxidation of aldophosphamide to carboxyphosphamide, but, with all other substrates, the specific activity of this enzyme exceeded that of the MCF-7/OAP enzyme even though K_m values

Table 6. Cofactor preferences of purified class 3 aldehyde dehydrogenases originally present in human MCF-7/OAP cells and stomach mucosa*

	MCF-7/OAP			Stomach mucosa		
Cofactor (mM)	K_m (μM)	V_{max} (mIU/mg)	V_{max}/K_m (mIU/mg/μM)	K_m (μM)	V_{max} (mIU/mg)	V_{max}/K_m (mIU/mg/μM)
NAD (0.02–1.0)	550	22,297	41	54	26,594	492
NADP (0.1–4.0)	940	37,133	40	1,000	80,160	80

* Kinetic constants were determined as described in Materials and Methods. Stock purified enzyme preparations were in Buffer B and were added to the reaction mixture in a volume of 100 μL. Benzaldehyde (4 mM) was the substrate. Each value is the mean of three determinations.

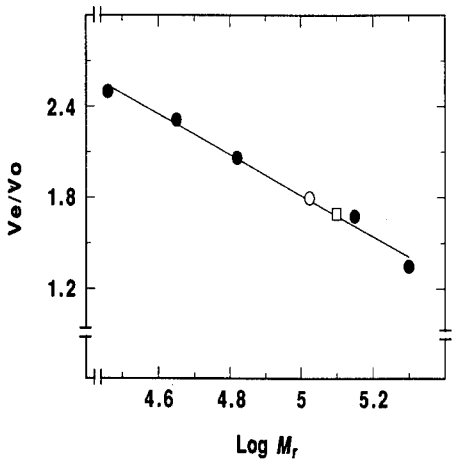

Fig. 10. Molecular weight determination of class 3 aldehyde dehydrogenases by gel permeation chromatography on Sephacryl S-200. Native molecular weights of the two enzymes were determined by gel permeation chromatography as described in Materials and Methods. Molecular weight markers (●) were carbonic anhydrase (29 kDa), ovalbumin (45 kDa), BSA monomer (66 kDa), alcohol dehydrogenase (141 kDa) and β-amylase (200 kDa). Marker proteins and aldehyde dehydrogenases (100 μg of each) were placed on the gel and elution was monitored at 280 nm. Elution of MCF-7/OAP (□) and stomach mucosa (○) aldehyde dehydrogenases was also monitored by measuring enzyme activity in the eluate fractions; substrate and cofactor were benzaldehyde (4 mM) and NADP (4 mM), respectively. V_e/V_0 = elution volume/void volume.

Fig. 11. Isoelectric focusing and immunoblot visualization of native stomach mucosa and MCF-7/OAP class 3 aldehyde dehydrogenases with anti-stomach mucosa ALDH-3 IgY. Anti-stomach mucosa ALDH-3 IgY was generated and used as described in Materials and Methods to visualize the purified stomach mucosa (lane 1) and MCF-7/OAP (lane 2) enzymes after they had been electrophoresed under non-denaturing conditions and electrotransferred onto Immobilon-PVDF transfer membranes. Placed on gels were 10 μg of each purified enzyme.

did not differ appreciably. The K_m value that we obtained for NAD-dependent stomach mucosa enzyme-catalyzed oxidation of benzaldehyde is similar to those reported by others [26, 27]. The MCF-7/OAP enzyme did catalyze the oxidation of aldophosphamide to carboxyphosphamide, albeit relatively poorly and then only when NAD was used as the cofactor. Glutamic-γ-semialdehyde (500 μM), succinic semialdehyde (100 μM), betaine aldehyde (100 μM), glyceraldehyde 3-phosphate (10 μM) and γ-aminobutyraldehyde (100 μM) were not substrates for either enzyme (data not presented).

As judged by K_m values, each of the enzymes preferred NAD as cofactor (Table 6). The K_m values reported herein for NAD and NADP (stomach mucosa enzyme-catalyzed oxidation of benzaldehyde) are not markedly different from those reported by others [26, 27]. Striking was the low K_m value, viz. 54 μM, obtained for NAD-dependent catalysis of benzaldehyde by the stomach mucosa enzyme as compared to that, viz. 550 μM, obtained for NAD-dependent catalysis of this substrate by the MCF-7/OAP enzyme. Cofactor inhibition was observed in the case of the MCF-7/OAP enzyme when the NAD concentration exceeded 1.0 mM. In contrast, cofactor "activation" was observed in the case of stomach mucosa enzyme when this concentration of NAD was exceeded (Fig. 17). High concentrations of NADP did not inhibit or "activate" either enzyme.

Each of the enzymes also exhibited esterolytic activity (Table 7). In each case, catalysis of *p*-nitrophenyl acetate hydrolysis was enhanced by 20 μM NAD and partially inhibited by 100 μM NAD. Inhibition of rat liver cytosol class 3 aldehyde dehydrogenase-catalyzed hydrolysis of *p*-nitrophenyl acetate by 100 μM NAD has been reported previously [28]. However, these investigators did not observe enhancement or inhibition of the reaction by 20 μM NAD. In contrast, Takahashi and Weiner [16] observed an enhancement of horse liver class 2 aldehyde dehydrogenase-catalyzed hydrolysis of *p*-nitrophenyl acetate by concentrations of NAD ranging from 120 to 200 μM.

The influence of agents known to enhance or inhibit the catalytic activity of various aldehyde dehydrogenases, on the ability of the two enzymes purified in this investigation to catalyze the oxidation of benzaldehyde is summarized in Table 8. Takahashi and Weiner [29] have shown that Mg^{2+} (250 and 500 μM) enhances the catalytic activity of a horse liver class 2 aldehyde dehydrogenase. Others have reported that these concentrations of Mg^{2+} do not enhance the catalytic activity of either a rat class 3

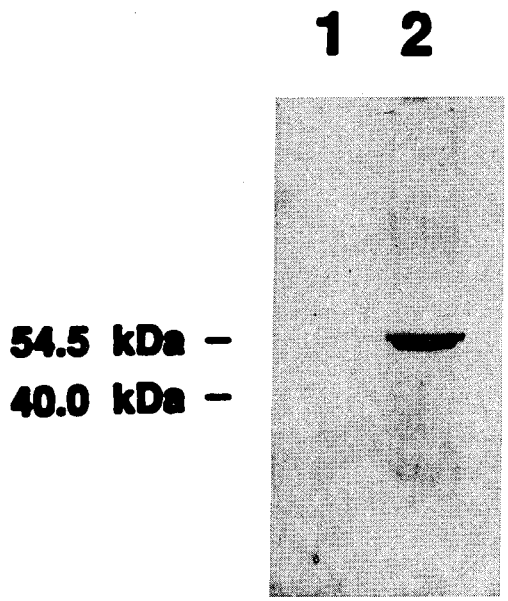

Fig. 12. SDS–PAGE and attempted immunoblot visualization of denatured stomach mucosa and MCF-7/OAP class 3 aldehyde dehydrogenases with anti-stomach mucosa ALDH-3 IgY. Anti-stomach mucosa ALDH-3 IgY was generated and used as described in Materials and Methods in an attempt to visualize the purified MCF-7/OAP (lane 1) and stomach mucosa (lane 2) enzymes after they had been electrophoresed under denaturing conditions and electrotransferred onto Immobilon-PVDF transfer membranes. Placed on the gels were 25 μg of each purified enzyme.

aldehyde dehydrogenase [28] or human stomach mucosa ALDH-3 [26]. In the present investigation, Mg^{2+} (250 or 500 μM) did not enhance the catalytic activity of either the human stomach mucosa, or the MCF-7/OAP, enzyme. Both enzymes were inhibited by *p*-chloromercuribenzoate; enzymes with a cysteine

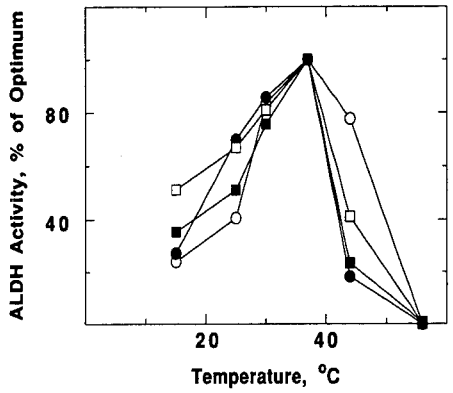

Fig. 13. Effect of temperature on the catalytic activity of purified stomach mucosa and MCF-7/OAP class 3 aldehyde dehydrogenases. Enzyme activity was determined as described in Materials and Methods using benzaldehyde (4 mM) as the substrate and NAD (1 mM) or NADP (4 mM) as cofactor. Optimal rates were at 37° and were 31 (NAD, ■) and 62 (NADP, □) nmol/min for stomach ALDH-3, and 2.7 (NAD, ●) and 5.9 (NADP, ○) nmol/min for MCF-7/OAP ALDH-3.

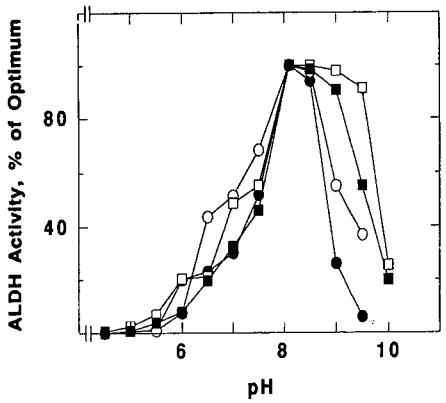

Fig. 14. Effect of pH on the catalytic activity of purified stomach mucosa and MCF-7/OAP class 3 aldehyde dehydrogenases. Enzyme activity was determined as described in Materials and Methods using buffers of different pH, benzaldehyde (4 mM) as substrate, and NAD (1 mM) or NADP (4 mM) as cofactor. Buffers were 32 mM sodium acetate (pH 4.5 to 5.5), 32 mM sodium pyrophosphate (pH 6.0 to 8.1) and 32 mM Tris–HCl (pH 8.5 to 10). Optimal rates were at pH 8.1 and were 33 (NAD, ■) and 62 (NADP, □) nmol/min for stomach ALDH-3, and 4.3 (NAD, ●) and 8.4 (NADP, ○) nmol/min for MCF-7/OAP ALDH-3.

residue at the catalytic site are typically sensitive to inhibition by this agent. The MCF-7/OAP enzyme was unaffected by 25 or 50 μM disulfiram; the stomach enzyme was inhibited slightly by these concentrations of disulfiram. Neither enzyme was affected by chloral hydrate.

ALDH-3 was also found in normal breast tissue obtained from either pre- or post-menopausal women (Fig. 18). In agreement with the findings of others [4], it was also detected in lung tissue but not in liver, kidney or placenta. The normal breast enzyme isoelectric-focused as did the MCF-7/OAP enzyme; the lung enzyme isoelectric-focused as did the stomach mucosa enzyme.

Quantitatively, and as expected given the foregoing observations, the ability of normal tissue to catalyze the oxidation of benzaldehyde was highest in stomach mucosa and was relatively high in lung (Table 9). It was, however, even higher in the malignant MCF-7/OAP cells. Assuming that 1×10^9 cells weigh 1 g, it can be calculated from the data presented in Table 2 that NAD- and NADP-dependent aldehyde dehydrogenase-catalyzed oxidation of benzaldehyde occurred at rates of 11,000 and 25,400 mIU/g, respectively. This type of a calculation reveals that, of the tissues examined, MCF-7/OAP cells were second only to the liver in being able to catalyze the oxidation of aldophosphamide to carboxyphosphamide, the calculated rate for MCF-7/OAP cells being 280 mIU/g.

DISCUSSION

The data reported herein clearly demonstrated that *associated* with the oxazaphosphorine-specific acquired resistance exhibited by the MCF-7/OAP cell line is a markedly elevated level of ALDH-3,

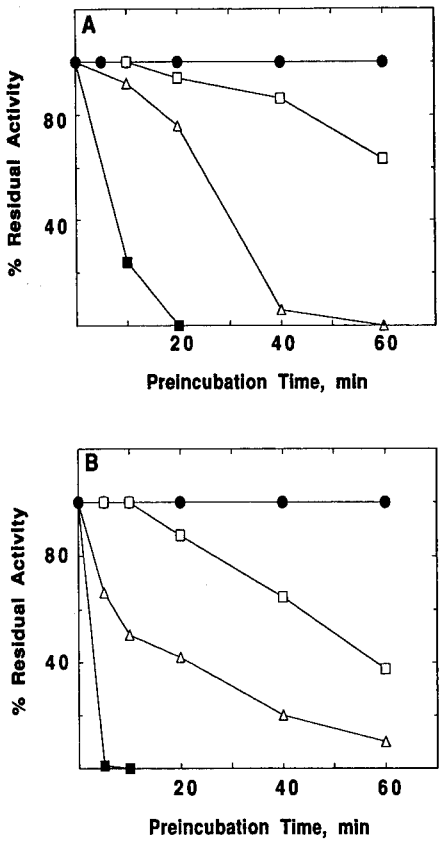

Fig. 15. Thermal stability of purified stomach mucosa and MCF-7/OAP class 3 aldehyde dehydrogenases. Enzymes were placed in Buffer B and were preincubated at 25° (●), 37° (□), 44° (△) or 56°(■) for the time periods indicated. At the end of the preincubation period, enzyme suspensions were rapidly cooled in an ice-bath, and enzyme activity was determined as described in Materials and Methods using benzaldehyde (4 mM) as substrate and NADP (4 mM) as cofactor. Control rates were 70 and 6.5 nmol/min for the stomach mucosa and MCF-7/OAP enzymes, respectively. Panel A, the MCF-7/OAP enzyme. Panel B, the stomach mucosa enzyme.

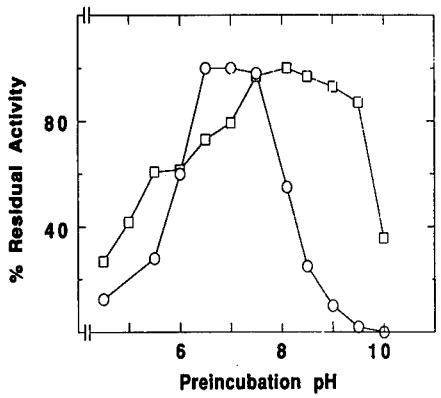

Fig. 16. Effect of pH on the stability of purified stomach mucosa and MCF-7/OAP class 3 aldehyde dehydrogenases. The purified stomach mucosa (□) and MCF-7/OAP (○) enzymes were preincubated at 4° for 24 hr in buffers adjusted to pHs ranging from 4.5 to 10. Buffers were 32 mM sodium acetate (pH 4.5 to 5.5), 32 mM sodium pyrophosphate (pH 6.0 to 8.1) and 32 mM Tris–HCl (pH 8.5 to 10). At the end of the 24-hr preincubation, pHs were readjusted to 8.1, and enzyme activity was determined as described in Materials and Methods using benzaldehyde (4 mM) as substrate and NADP (4 mM) as cofactor. Control rates were 52 and 7 nmol/min for the stomach mucosa and MCF-7/OAP enzymes, respectively.

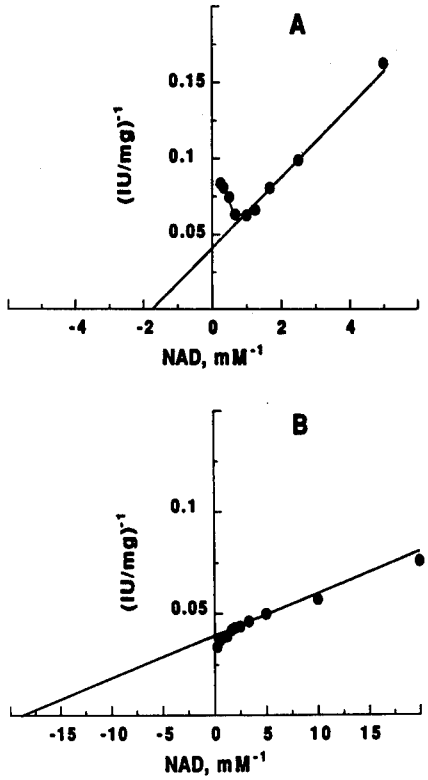

Fig. 17. Catalysis of benzaldehyde (4 mM) oxidation by purified MCF-7/OAP (A), and human stomach mucosa (B), ALDH-3 as a function of NAD concentration: Lineweaver–Burk kinetic analysis. Initial rates were determined as described in Materials and Methods. K_m and V_{max} values were calculated from x- and y-intercepts, respectively, and are given in Table 6. Each point is the mean of triplicate determinations.

and, in aggregate, led us to conclude that whereas the MCF-7/OAP enzyme, as well as the MCF-7/0 and normal breast counterparts, is a cytosolic class 3 aldehyde dehydrogenase, it is somewhat different from the previously characterized stomach mucosa ALDH-3 and hence a novel ALDH-3. Thus, the breast enzyme is referred to hereafter in this paper as Type-2 ALDH-3 to distinguish it from the stomach mucosa enzyme referred to hereafter in this paper as Type-1 ALDH-3. The molecular basis for the overexpression of Type-2 ALDH-3 by MCF-7/OAP cells is not known but is being investigated currently in our laboratory.

Type-2 ALDH-3 catalyzed the oxidation of aldophosphamide, albeit poorly, whereas Type-1 ALDH-3 did not. AHD-4, the class 3 aldehyde dehydrogenase found in the cytosol of mouse stomach mucosa may be, somewhat unexpectedly, the mouse homolog of human breast Type-2 ALDH-

Table 7. Esterase activity of purified class 3 aldehyde dehydrogenases present in human MCF-7/OAP cells and stomach mucosa*

NAD (μM)	v (mIU/mg)	
	MCF-7/OAP	Stomach mucosa
0	3,350†	9,800†
20	4,500	11,000
100	2,500	8,500

* The rate at which purified class 3 aldehyde dehydrogenases catalyzed the hydrolysis of *p*-nitrophenyl acetate (500 μM) to *p*-nitrophenol was determined as described in Materials and Methods. Stock purified enzyme preparations were in Buffer B and were added to the reaction mixture (final volume = 3 mL) in a volume of 200 μL. Each value is the mean of three determinations, each made in duplicate.

† NADP-dependent (4 mM) MCF-7/OAP and stomach mucosa ALDH-3-catalyzed oxidation of benzaldehyde (V_{max} values, Table 5) was about nine and five times faster, respectively.

3 rather than of human stomach mucosa Type-1 ALDH-3 since it also catalyzes the oxidation of aldophosphamide [2]. Mouse hepatic AHD-7 has also been putatively identified as a cytosolic class 3 aldehyde dehydrogenase [2, 30]. It does not catalyze the oxidation of aldophosphamide and may be the mouse homolog of human Type-1 ALDH-3.

Given that aldophosphamide is a relatively poor substrate for Type-2 ALDH-3, the notion that increased Type-2 ALDH-3-catalyzed oxidation of aldophosphamide accounts for the oxazaphosphorine-specific acquired resistance exhibited by the MCF-7/OAP cell line remains highly problematical. Perhaps Type-2 ALDH-3 accounts for this resistance in some other, as yet unidentified or even anticipated, way. Strongly supporting the notion that Type-2 ALDH-3 in some way accounts for the oxazaphosphorine-specific acquired resistance exhibited by MCF-7/OAP cells are the observations that inclusion of aldehydes that are good substrates for this enzyme, viz. benzaldehyde, 4-pyridinecarboxaldehyde or octanal, in the drug-exposure medium largely restored the sensitivity of the MCF-7/OAP cells to mafosfamide whereas inclusion of a poor substrate, viz. acetaldehyde, did not, that inclusion of benzaldehyde, 4-pyridinecarboxaldehyde or octanal in the drug-exposure medium only slightly increased the sensitivity of MCF-7/0 cells to mafosfamide, and that sensitivity to phosphoramide mustard on the part of either MCF-7/0 or MCF-7/OAP cells was largely unaffected by any of these aldehydes.*

It follows that Type-1 ALDH-3 may also be an important determinant of cellular sensitivity to the

* Sladek NE and Sreerama L, Manuscript in preparation.

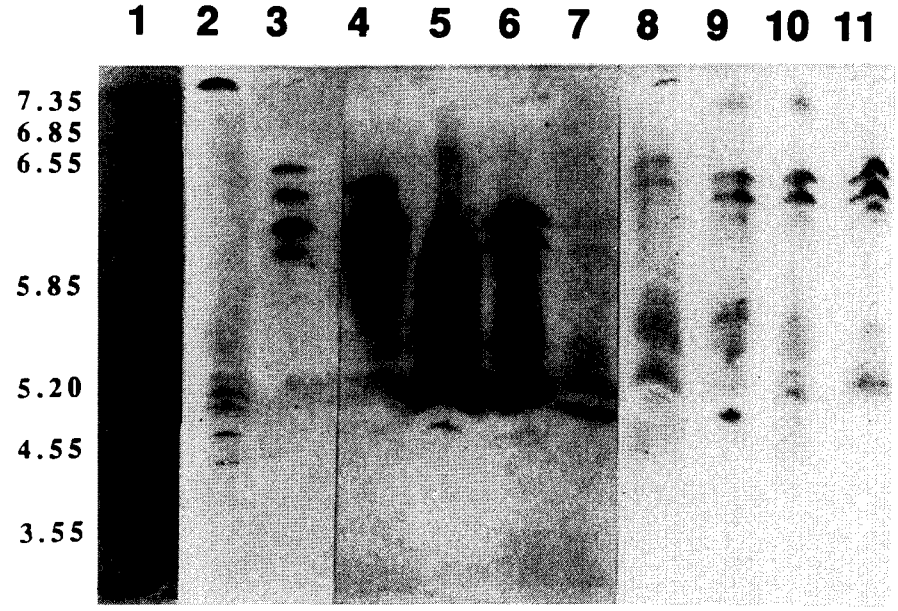

Fig. 18. Isoelectric focusing of aldehyde dehydrogenases present in various human tissues. Lubrol®-treated whole homogenates of liver, kidney, and 2.5×10^5 MCF-7/OAP cells, and soluble (105,000 *g* supernatant) fractions of stomach mucosa, lung, placenta and 1 g of normal breast, were electrofocused as described in Materials and Methods. Placed on the gel were the amounts of tissue preparation indicated above or an amount of tissue preparation sufficient to generate 5.0 nmol NADH/min when 4 mM acetaldehyde (liver, placenta) or 4 mM benzaldehyde (kidney, stomach mucosa, lung) was used as the substrate. Lane 1, pI standards; lane 2, liver; lane 3, MCF-7/OAP cells; lane 4, stomach mucosa; lane 5, kidney; lane 6, lung; lane 7, placenta; lane 8, breast from a 61-year-old female; lane 9, breast from a 59-year-old female; lane 10, breast from a 51-year-old female; and lane 11, breast from a 19-year-old female. Lane 1 was stained for protein with Coomassie Brilliant Blue R-250, and lanes 2–11 were stained for aldehyde dehydrogenase activity using benzaldehyde (4 mM) as substrate and NAD (4 mM) as cofactor, as described in Materials and Methods.

Table 8. Influence of various effectors on the catalytic activity of human class 3 aldehyde dehydrogenases isolated from MCF-7/OAP cells and stomach mucosa*

Effector	Cofactor	Concentration of effector (μM)	% Control aldehyde dehydrogenase activity	
			MCF-7/OAP	Stomach mucosa
Disulfiram	NAD	25	98	75
		50	99	70
	NADP	25	100	80
		50	98	70
p-Chloromercuribenzoate	NAD	10	87	89
		25	4	7
	NADP	10	75	91
		25	2	3
Chloral hydrate	NAD	50	99	100
		1000	96	93
	NADP	50	100	98
		1000	95	96
Mg^{2+}	NAD	250	96	100
		500	90	99
	NADP	250	87	98
		500	87	89

* Purified enzymes were transferred from Buffer B into 25 mM sodium phosphate buffer, pH 7.5, with the aid of a PD-10 column prior to assay, and aldehyde dehydrogenase activities were measured as described in Materials and Methods except that the complete reaction mixture, minus the substrate, was preincubated at 37° for 5 min. Benzaldehyde (4 mM) was the substrate and NAD (1 mM) or NADP (4 mM) was the cofactor. Glutathione was omitted from the reaction mixture when disulfiram, *p*-chloromercuribenzoate and chloral hydrate were tested; EDTA was left out when Mg^{2+} was tested. Control rates ranged from 5.0 to 5.5 (NAD) and 8.5 to 9.0 (NADP) nmol/min for MCF-7/OAP aldehyde dehydrogenase, and from 26 to 27 (NAD) and 58 to 59 (NADP) nmol/min for stomach mucosa aldehyde dehydrogenase. Values are the means of triplicate determinations.

oxazaphosphorines although this possibility is even more remote since Type-1 ALDH-3 does not catalyze the oxidation of aldophosphamide, and an inverse relationship between Type-1 ALDH-3 levels and cellular sensitivity to oxazaphosphorines has yet to be reported. Nevertheless, the idea is attractive, especially since the Type-1 enzyme may have a much wider tissue distribution than does the Type-2 enzyme [4]. Indeed, at this time, the possibility that the Type-2 enzyme is a tissue-specific, viz. breast, enzyme cannot be dismissed. Relevant also is that Type-1 ALDH-3 is not found in all normal tissue nor is it found in all malignant tissue [4, 25]. Whether cells that ordinarily do not express ALDH-3 (even at low levels) can be caused to do so on a permanent basis by exposing them to a mutagen such as one

Table 9. Aldehyde dehydrogenase activity in selected human tissues*

Substrate (mM)	Cofactor (4 mM)	Aldehyde dehydrogenase activity (mIU/g tissue)					
		Breast	Stomach mucosa	Lung	Placenta	Kidney	Liver
Benzaldehyde (4.0)	NAD	24	4615	1495	8	360	610
	NADP	6	8750	3270	1	34	567
Acetaldehyde (4.0)	NAD	68	2406	407	26	197	6642
	NADP	0	2150	344	0	0	1417
Aldophosphamide (0.16)	NAD	36	63	44	11	52	1295
	NADP	0	0	0	0	0	ND†
Glutamic-γ-semialdehyde (0.5)	NAD	0	0	0	0	1880	701
	NADP	0	0	0	0	0	ND

* Preparation of soluble (105,000 *g* supernatant) fractions from breast, stomach mucosa, lung and placenta, and Lubrol®-treated whole homogenates of kidney and liver, and determination of the rate at which these preparations catalyzed the oxidation of various aldehydes to their corresponding acids were as described in Materials and Methods. Except in the case of breast tissue, each value is the mean of duplicate determinations made three different times on the same tissue sample. The mean of duplicate determinations made once on each of four tissue samples is reported for breast tissue.

† ND: not determined.

of the oxazaphosphorines, also remains to be determined.

Seemingly at odds with any suggestion that increases in ALDH-3 account for decreases in sensitivity to the oxazaphosphorines is the lack of any inverse correlation between sensitivity to the oxazaphosphorines on the part of several hepatoma cell lines and expression of a cytosolic class 3 aldehyde dehydrogenase by these cell lines reported by Lin and Lindahl [31]. A shortcoming of these experiments in that regard, however, is that the relative sensitivity of each of these cell lines to non-oxazaphosphorine cross-linking agents was not determined so the values quantifying oxazaphosphorine-sensitivity could not be normalized for nonspecific determinants of cellular sensitivity to the oxazaphosphorines, i.e. values quantifying sensitivity to the oxazaphosphorines were influenced by both oxazaphosphorine-specific, e.g. metabolism, and -nonspecific, e.g. nonspecific binding sites, determinants of sensitivity.

The foregoing deliberations are potentially of substantial clinical significance in view of the extensive use of cyclophosphamide as part of the therapeutic protocol in the treatment of breast cancer and the unacceptably high relapse (resistance) rate that neoplasms treated with such protocols exhibit.

Multiple bands were observed when either Type-1 or Type-2 ALDH-3 was isoelectric-focused; pI values for three of the bands obtained with the Type-2 enzyme were identical with the pI values, viz. 6.0, 6.25 and 6.35, obtained with the Type-1 enzyme. However, one of the bands (pI = 6.45) seen with the Type-2 enzyme was not seen with the Type-1 enzyme, and two of the bands (pI = 5.75 and 5.85) seen with the Type-1 enzyme were not seen with the Type-2 enzyme. Moreover, the relative amounts of the common bands differed substantially.

It has been established that a single gene codes for the synthesis of human stomach mucosa ALDH-3 [32]. This gene is found in chromosome 17 [33], is about 8 kb in length, and is interspersed with 10 exons. Whether the polymorphism exhibited by human stomach mucosa ALDH-3 is due to transcriptional, translational, and/or *in vivo* or *in vitro* post-translational events, e.g. proteolytic digestion [34], is unknown. Similarly, the basis for the unique polymorphism exhibited by the MCF-7/OAP enzyme is unknown. The presence of a protease inhibitor during enzyme purification did not alter the isoelectric focusing banding patterns of either enzyme; "new" bands of catalytic activity did not appear when either of the two enzymes was stored under conditions that allowed for some loss of gross catalytic activity.

ALDH-3 is one of at least six "drug-metabolizing enzymes" that are coded for by genes that are members of the so-called polycyclic aromatic hydrocarbon-responsive gene battery [25]. The others are cytochrome P450s IA1 and IA2, glutathione *S*-transferase, NAD(P)H:menadione oxidoreductase (DT-diaphorase) and UDP-glucuronyl transferase. Activation of the gene battery is believed to involve a series of receptor-mediated events initiated by the binding of an appropriate agonist (ligand) to a cytosolic protein termed the Ah receptor [25]. Known agonists for the Ah receptor include the polycyclic aromatic hydrocarbons, 3-methylcholanthrene, benzpyrene, 9,10-dimethyl-1,2-benzanthracene and, especially, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) [25]. One or more of these agents have been shown to induce ALDH-3 in rat and human hepatocytes and hepatoma cells [35–37]. Moreover, polycyclic aromatic hydrocarbons are known to induce cytochrome P450 IA1 in MCF-7 cells [38]. Thus, it seemed likely that these agents would also induce the expression of ALDH-3 in these cells. This expectation has been realized [39].

An endogenous substrate for ALDH-3 has yet to be identified and its biological role is unknown. Perhaps its *raison d'etre* is to detoxify xenobiotics. Supporting this notion are the observations that tissues which come in direct contact with xenobiotics present in food, water and air, e.g. stomach mucosa, intestinal mucosa and lungs, contain large amounts of this enzyme [4]. Apparently inconsistent with this notion is the high level of ALDH-3 in the cornea although a similar role, viz. protection against UV-light-induced cellular damage, has been proposed for ALDH-3 in this tissue [40]. Alternatively, ALDH-3 levels, as well as the levels of other products of the Ah receptor/agonist-activated gene battery, may be high in tissues that constitute "ports of entry" because they come in direct contact with relevant inducing agents that are present in the environment. The elevated ALDH-3 levels may then serve to protect such cells from any further damage by these agents. Along these lines, Yin *et al.* [41] reported that ALDH-3 could not be found in 56% of lung tissue samples obtained from Chinese adults, but low levels were found in some (34%) and relatively high levels were found in the remainder (10%). Attractive is the notion that lung tissue ALDH-3 levels reflect the exposure of this tissue to polycyclic aromatic hydrocarbons, e.g. those in cigarette smoke.

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Identification of a Methylcholanthrene-induced Aldehyde Dehydrogenase in a Human Breast Adenocarcinoma Cell Line Exhibiting Oxazaphosphorine-specific Acquired Resistance[1]

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ABSTRACT

The class-3 aldehyde dehydrogenase that is overexpressed (>100-fold) in human breast adenocarcinoma MCF-7/0 cells made resistant (>30-fold as judged by LC_{90}s) to oxazaphosphorines, such as mafosfamide, by growing them in the presence of polycyclic aromatic hydrocarbons, *e.g.*, methylcholanthrene (3 μM for 5 days), was isolated and characterized. Its physical and catalytic properties were identical to those of the prototypical human stomach mucosa cytosolic class-3 aldehyde dehydrogenase, type-1 ALDH-3, except that it catalyzed, though not very rapidly, the oxidation of aldophosphamide, whereas the stomach mucosa enzyme essentially did not; hence, it was judged to be a slight variant of the prototypical enzyme. Carcinogens that are not ligands for the Ah receptor, barbiturates known to induce hepatic cytochrome P450s, steroid hormones, an antiestrogen, and oxazaphosphorines did not induce the enzyme or the largely oxazaphosphorine-specific acquired resistance. Whereas methylcholanthrene induced (*a*) resistance to mafosfamide and (*b*) class-3 aldehyde dehydrogenase activity, as well as glutathione *S*-transferase and DT-diaphorase activities, in the estrogen receptor-positive MCF-7/0 cells, it did not do so in two other human breast adenocarcinoma cell lines, MDA-MB-231 and SK-BR-3, each of which is estrogen receptor negative. Expression of the class-3 aldehyde dehydrogenase and the loss of sensitivity to mafosfamide by polycyclic aromatic hydrocarbon-treated MCF-7/0 cells were transient; each returned to essentially basal levels within 15 days when the polycyclic aromatic hydrocarbon was removed from the culture medium. Insensitivity to the oxazaphosphorines on the part of polycyclic aromatic hydrocarbon-treated MCF-7/0 cells was not observed when exposure to mafosfamide (30 min) was in the presence of benzaldehyde or octanal, each a relatively good substrate for cytosolic class-3 aldehyde dehydrogenases, whereas it was retained when exposure to mafosfamide was in the presence of acetaldehyde, a relatively poor substrate for these enzymes. These observations demonstrate that ligands for the Ah receptor can induce a transient, largely oxazaphosphorine-specific, acquired cellular resistance, and they are consistent with the notion that elevated levels of a cytosolic class-3 aldehyde dehydrogenase nearly identical to the prototypical type-1 class-3 aldehyde dehydrogenase expressed by human stomach mucosa account for the Ah receptor ligand-induced oxazaphosphorine-specific acquired resistance, most probably by catalyzing the detoxification of aldophosphamide.

INTRODUCTION

Although cyclophosphamide and other oxazaphosphorines, *e.g.*, ifosfamide, 4-hydroperoxycyclophosphamide, and mafosfamide, are amongst the more useful antitumor agents currently available, total eradication of tumor cells with these agents, *i.e.*, cures, is seldom achieved. This is often because the target cells are intrinsically insensitive to these prodrugs or because they acquire insensitivity to them. It follows, then, that the identification of the cellular determinants of tumor sensitivity to the oxazaphosphorines, especially those specific for these agents, and thus the potential biochemical bases for intrinsic and acquired cellular resistance to the oxazaphosphorines, is of importance if the future use of these drugs is to be optimized and if more useful drugs of this type are to be designed.

One such determinant, a class-1 aldehyde dehydrogenase termed ALDH-1 in humans and AHD-2 in mice, has already been identified (reviewed in Refs. 1 and 2). This enzyme catalyzes the irreversible detoxification of the oxazaphosphorines, specifically, the oxidation of aldophosphamide (aldoifosfamide), the pivotal metabolite of the oxazaphosphorines, to carboxyphosphamide (carboxyifosfamide). Thus, sensitivity to the oxazaphosphorines decreases as the level of this enzyme increases.

A cytosolic class-3 aldehyde dehydrogenase termed ALDH-3 may also be an important cellular determinant of tumor sensitivity to the oxazaphosphorines. Thus, we have shown (3) that ALDH-3 activity is markedly elevated (>100-fold) in human MCF-7 breast adenocarcinoma cells made resistant to the oxazaphosphorines by growing the parent MCF-7/0 line in the presence of increasing concentrations of 4-hydroperoxycyclophosphamide for several months (4). Resistance was apparently stable (for at least 2 months in the absence of any selecting pressure), substantial (>40-fold loss of sensitivity as judged by LC_{90}s[3] for mafosfamide), and largely oxazaphosphorine-specific, *i.e.*, the oxazaphosphorine-resistant subline, termed MCF-7/OAP, was only minimally cross-resistant to phosphoramide mustard and several other cytotoxic agents (1.3- to 3.1-fold as judged by LC_{90}s). Suggesting that elevated levels of ALDH-3 accounted for the oxazaphosphorine-specific resistance were the observations that inclusion of aldehydes that are good substrates for cytosolic class-3 aldehyde dehydrogenase (benzaldehyde, octanal, and 4-pyridinecarboxaldehyde) in the drug exposure medium restored the sensitivity of MCF-7/OAP cells to mafosfamide, whereas inclusion of acetaldehyde, an aldehyde that is a poor substrate for this enzyme, did not.[4] At odds with this notion was the observation that, as judged by the apparent K_m value, aldophosphamide was seemingly a poor substrate for the ALDH-3 purified from the resistant MCF-7/OAP cells. It was also established that, although a class-3 aldehyde dehydrogenase, the ALDH-3 present in MCF-7/OAP cells was somewhat different from the previously characterized prototypical human stomach mucosa ALDH-3 and hence, an apparently novel ALDH-3. The novel enzyme was christened type-2 ALDH-3 to distinguish it from the prototypical enzyme which was referred to as type-1 ALDH-3. Whereas type-2 ALDH-3 catalyzed the oxidation of aldophosphamide to carboxyphosphamide, albeit seemingly poorly, the prototypical type-1 ALDH-3 essentially did not.

Directly relevant to the present investigation, ALDH-3 activity was also markedly elevated (>100-fold) in MCF-7/0 cells exposed to

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[3] The abbreviations used are: LC_{90}, drug concentration required to effect a 90% cell kill; mIU, milli-International Unit of enzyme activity (nmol NAD(P)H formed/min in the case of aldehyde dehydrogenase activity, nmol of the conjugate of 1-chloro-2,4-dinitrobenzene and glutathione formed/min in the case of glutathione *S*-transferase activity, and nmol of 2,6-dichlorophenol-indophenol reduced/min in the case of DT-diaphorase activity); pI, isoelectric point; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate.

[4] L. Sreerama and N. E. Sladek, unpublished observations.

various polycyclic aromatic hydrocarbons, *e.g.*, methylcholanthrene and 3,4-benzpyrene, for a few days (5). Termed MCF-7/MC, methylcholanthrene-treated cells also exhibited substantial, largely oxazaphosphorine-specific, acquired resistance (>30-fold loss of sensitivity as judged by LC_{90}s for mafosfamide; resistance to phosphoramide mustard was only 1.7-fold as judged by LC_{90}s; Ref. 5). Oxazaphosphorine-specific acquired resistance of an identical magnitude was obtained when MCF-7/0 cells were exposed to 3 μM 3,4-benzpyrene for 5 days.[4] ALDH-3 levels were only transiently elevated, *i.e.*, they returned to basal levels within 15 days after removal of methylcholanthrene from the incubation medium in which the tumor cells were grown (5).

Not determined in the foregoing investigation was whether the induced ALDH-3 was of the type-1 or type-2 variety. Also not determined was whether oxazaphosphorine-specific resistance was, like the elevated ALDH-3 activity, transient.

These determinations were made in the present investigation. Also investigated was the ability of: (*a*) benzaldehyde, octanal, and acetaldehyde to restore the sensitivity of MCF-7/MC cells to mafosfamide; (*b*) certain other agents to induce ALDH-3 activity in MCF-7/0 cells; and (*c*) polycyclic aromatic hydrocarbons to induce ALDH-3 activity and/or oxazaphosphorine-specific resistance in other breast cancer cell lines.

MATERIALS AND METHODS

Materials. Mafosfamide and 4-hydroperoxycyclophosphamide were provided by Dr. J. Pöhl, Asta-Werke AG, Bielefeld, Germany. Cyclophosphamide was a generous gift from Dr. W. A. Zygmunt, Mead Johnson Research Center, Evansville, IN. Methyl sulfide (99+%) and dimethyl sulfoxide (99+%) were purchased from the Aldrich Chemical Company, Milwaukee, WI. Glutathione reductase, flavin-adenine dinucleotide, 2,6-dichlorophenol-indophenol, Tween 20 (polyoxyethylenesorbitan monolaurate), 3-methylcholanthrene, 3,4-benzpyrene, 9,10-dimethyl-1,2-benzanthracene, 2-acetylaminofluorene, diethylnitrosamine, ethionine, phenobarbital, barbital (5,5′-diethylbarbituric acid), and sodium pyruvate (cell culture grade) were purchased from Sigma Chemical Company, St. Louis, MO. Horse (equine) serum was purchased from Hyclone Laboratories, Logan, UT. Precast polyacrylamide gradient (4–20%) gels (Mini-PROTEAN II ready gels) were purchased from Bio-Rad Laboratories, Richmond, CA. Basal medium Eagle amino acids (100X) and vitamin (100X) solutions, penicillin (10,000 units/ml)-streptomycin (10,000 mcg/ml) solution, and McCoy's 5A (modified) medium (powder; high glucose) were purchased from Gibco Laboratories, Grand Island, NY. 17-β-Estradiol, tamoxifen citrate, and progesterone were purchased from ICN Biochemicals Inc., Irvine, CA. Androsterone and corticosterone were purchased from Fluka Chemical Corporation, Ronkonkoma, NY. All other chemicals and reagents were obtained from the sources reported in a previous publication (3).

Aldophosphamide was generated in aqueous solution by chemical reduction of 4-hydroperoxycyclophosphamide using methyl sulfide as the reducing agent (3, 6). 4-Hydroperoxyifosfamide was prepared for us from ifosfamide by Dr. Kathleen Getman, University of Minnesota, Minneapolis, MN, according to the protocol described by Peter *et al.* (7). Human stomach mucosa type-1 ALDH-3 and human breast adenocarcinoma MCF-7/OAP type-2 ALDH-3 were purified from their respective sources as described previously (3). Antibodies against human stomach mucosa type-1 ALDH-3 were obtained by immunizing egg-laying hens (White Leghorn) with the enzyme as described previously (3).

Buffers A and B were 25 mM 2-(*N*-morpholino)ethane sulfonic acid, pH 6.5, and 25 mM sodium phosphate, pH 7.5, respectively, each supplemented with 1 mM EDTA and 0.05% dithiothreitol. Homogenization medium was 1.15% (w/v) KCl and 1 mM EDTA in aqueous solution, pH 7.4. Drug exposure medium was horse (fetal bovine serum in the case of SK-BR-3 cells) serum (10%) in a phosphate-buffered saline-based solution, pH 7.4, prepared as described previously (8).

Human MCF-7/0 and MCF-7/OAP breast adenocarcinoma cells were originally obtained from Dr. B. Teicher, Dana-Farber Cancer Institute, Boston, MA, and were cultured as described previously (3) except that 10% horse, rather than 10% bovine, serum was used. Human SK-BR-3 breast adenocarcinoma cells were provided by Dr. S. Ramakrishnan, Department of Pharmacology, University of Minnesota Medical School, Minneapolis, MN. They were cultured in McCoy's 5A (modified) medium supplemented with fetal bovine serum (10%), L-glutamine (2 mM), sodium bicarbonate(2.2 g/liter), basal medium Eagle amino acids solution (6 ml/liter), basal medium Eagle vitamin solution (6 ml/liter), sodium pyruvate (1 mM), penicillin (100,000 units/liter), and streptomycin (100,000 mcg/liter). Human MDA-MB-231 breast adenocarcinoma cells were purchased from American Type Culture Collection, Rockville, MD. They were cultured in Dulbecco's modified Eagle's medium supplemented with horse serum (10%), L-glutamine (2 mM), sodium bicarbonate (3.7 g/liter), gentamicin (50 mg/liter), basal medium Eagle amino acids solution (6 ml/liter), basal medium Eagle vitamin solution (6 ml/liter), and sodium pyruvate (1 mM). All of the cell lines were grown as monolayer cultures at 37°C as described previously (3). Mean population doubling times were approximately 25 (MCF-7/0), 40 (MDA-MB-231), and 45 (SK-BR-3) h. Mean plating efficiencies were approximately 50 (MCF-7/0), 20 (methylcholanthrene-treated MCF-7/0), 30 (MDA-MB-231), and 35 (SK-BR-3) %.

Cultured tumor cells in asynchronous exponential growth were harvested and checked for viability (usually greater than 95% as judged by trypan blue exclusion; preparations exhibiting less than 85% viability were discarded) as described previously (3). This was the preparation used in the colony-forming assays. Cells were further handled in two different ways when enzyme activity in cell-free fractions was to be quantified or when ALDH-3 was to be purified. They were reharvested by low-speed centrifugation (500 *g* for 10 min), resuspended (1×10^7 cells/ml) in homogenization medium, and then used when enzyme activity in cell-free fractions was to be quantified. They were also reharvested by low-speed centrifugation when ALDH-3 was to be purified but were stored at −20°C as a pellet overlaid with homogenization medium until used. Enzyme activity was essentially unaffected by freezing and storage at −20°C.

Induction of ALDH and Other Enzymes. Tumor cells were cultured for 6 days as described above except that potential inducers of enzyme activity were added to the growth medium after 1 day of growth. They were then harvested and further used as described below. Methylcholanthrene, 3,4-benzpyrene, 9,10-dimethyl-1,2-benzanthracene, ethionine, phenobarbital, barbital, 17-β-estradiol, tamoxifen citrate, progesterone, androsterone, and corticosterone were dissolved in dimethyl sulfoxide. Diethylnitrosamine, cyclophosphamide, 4-hydroperoxyifosfamide, and mafosfamide were dissolved in double-deionized water. Each of these stock solutions was diluted with growth medium and subsequently sterilized by passage through 0.22 μm Millipore filters; all were used within 2 h after initially being put into solution. Control cultures received vehicle (water or dimethyl sulfoxide diluted with growth medium) alone. The dimethyl sulfoxide concentration in the ultimate culture media never exceeded 0.1%; this concentration of dimethyl sulfoxide did not affect cell growth, cellular glutathione concentration, or aldehyde dehydrogenase, glutathione *S*-transferase, or DT-diaphorase activities.

Drug Exposure and Colony-forming Assay. Drug exposure and the colony-forming assay used to determine surviving fractions were as described previously (3, 5). Essentially, freshly harvested cells were diluted with drug exposure medium to a concentration of 1×10^5 cells/ml and then exposed to mafosfamide or vehicle for 30 min at pH 7.4 and 37°C, after which they were harvested and cultured in drug-free growth medium for 15 (MCF-7/0, MDA-MB-231, SK-BR-3, and methylcholanthrene-treated MDA-MB-231 and SK-BR-3 cells) to 30 (methylcholanthrene-treated MCF-7/0 cells) days. Colonies (≥50 cells) were then visualized with methylene blue dye and counted.

Preparation of Subcellular Fractions and Lubrol-treated Whole Homogenates. Subcellular (105,000 g soluble and solubilized 105,000 g pellet) fractions and Lubrol-treated whole homogenates were prepared as described previously (3).

Enzyme Assays. Aldehyde dehydrogenase and esterase activities were quantified spectrophotometrically as described previously (3).

Glutathione *S*-transferase activity was determined as described by Habig *et al.* (9). The reaction mixture (1 ml), pH 6.5, contained 100 mM potassium phosphate buffer, 1 mM glutathione, Lubrol-treated whole homogenates prepared from 1×10^5 to 5×10^6 cells, and 1 mM substrate (1-chloro-2,4-dinitrobenzene). The reaction was initiated by the addition of substrate, and

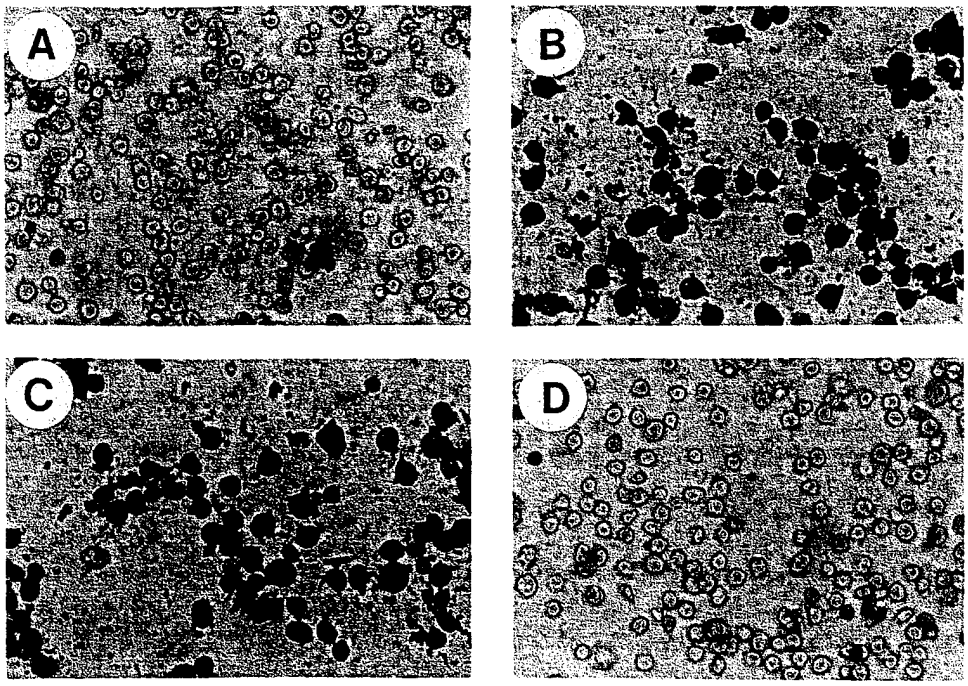

Fig. 1. Histochemical staining of MCF-7/0 cells incubated with 3 μM methylcholanthrene for aldehyde dehydrogenase activity. MCF-7/0 cells were harvested at various times and histochemically stained for aldehyde dehydrogenase activity as described in "Materials and Methods." *A*, before incubation with methylcholanthrene. *B*, after incubation with methylcholanthrene for 5 days. *C*, after incubation with methylcholanthrene for 5 days, followed by incubation for 3 days in methylcholanthrene-free medium. *D*, after incubation with methylcholanthrene for 5 days, followed by incubation for 9 days in methylcholanthrene-free medium.

formation of the glutathione conjugate was monitored at 340 nm and 25°C. All rates were determined in duplicate.

DT-Diaphorase activity was determined essentially as described by Benson *et al.* (10). The reaction mixture (1 ml), pH 7.4, contained 25 mM Tris-HCl buffer, 0.23 mg bovine serum albumin, 0.01% Tween 20, 5 μM flavin-adenine dinucleotide, 0.16 mM NADH, ± 10 μM dicumarol, Lubrol-treated whole homogenates prepared from 4×10^4 to 2.5×10^5 cells, and 40 μM substrate (2,6-dichlorophenol-indophenol). The reaction was started by the addition of substrate, and reduction of 2,6-dichlorophenol-indophenol was monitored at 600 nm and 25°C. All rates were determined in duplicate.

Determination of Glutathione Levels. The reductase recycling assay described by Anderson (11) was used to quantify cellular glutathione content. Cells (2.5×10^6–1×10^7) were homogenized in 10 mM HCl (100–400 μl) by sonication, and the homogenate was deproteinized by adding 50 μl of a 10% 5-sulfosalicylic acid in water solution. Protein-free supernatant solutions of the homogenates were obtained by centrifugation at 10,000 *g* and subsequently stored at 4°C until assayed. The assay mixture (1 ml; pH 7.5) contained 0.21 mM NADPH, 0.6 mM 5,5′-dithiobis-2-nitrobenzoic acid, 5 mM EDTA, 26 mIU glutathione reductase, 116 mM sodium phosphate, and 50 μl protein-free cell homogenate. The reaction mixture, except for the protein-free homogenate and glutathione reductase, was preincubated at 30°C for 10 min. Cell homogenate and glutathione reductase were then added, and the rate of thionitrobenzoic acid formation was monitored at 412 nm. Authentic glutathione was used to develop a standard curve.

Protein Determination. Protein concentrations were estimated by the method of Bradford (12). Bovine serum albumin was used as the standard.

Chromatographic Purification of Aldehyde Dehydrogenases. CM-Sepharose CL 6B, Reactive Blue 2-Sepharose CL 6B, and DEAE-Sephacel column chromatography was performed at 4 to 6°C essentially as described previously (3). All buffers were degassed prior to use. Samples were concentrated with the aid of an Amicon Diaflo concentrator fitted with a YM-30 membrane under nitrogen pressure. Protein concentrations of samples loaded onto columns never exceeded 15 mg/ml and usually were much less. Benzaldehyde (4 mM) and NAD (1 mM) were used as the substrate and cofactor, respectively, to monitor aldehyde dehydrogenase activity in column eluates. Protein was monitored at 280 nm with an ISCO UA-5 absorbance monitor. Purified enzymes could be stored frozen in Buffer B at −20°C for at least 4 months with little (<10%) loss of activity.

Analytical PAGE, Isoelectric Focusing, Immunoblot Analysis, and Molecular Weight Determinations. Each of these was as described previously (3) except that commercially available precast polyacrylamide gradient (4–20%) gels (mini-PROTEAN II ready gels) were used when the native molecular weight was determined by linear gradient gel electrophoresis.

Histochemical Staining for Aldehyde Dehydrogenase. The method of Lindahl *et al.* (13) was used, with slight modification, to histochemically visualize aldehyde dehydrogenase activity in intact MCF-7/0 cells. Low-speed centrifugation (500 *g* for 10 min) was used to harvest and wash 2×10^5 tumor cells. Staining solution (2.16 mg nitroblue tetrazolium and 0.18 mg phenazine methosulfate in 1.8 ml of an aqueous 32 mM sodium pyrophosphate buffer solution, pH 7.5) was added to the pelleted cells, and mild vortexing was used to put them back into suspension. This preparation was incubated at 37°C in the dark for 5 min in a shaking water-bath; benzaldehyde (4 mM) and NAD (4 mM) were added, incubation was continued for 10 min, and the reaction was stopped by adding 2 ml of an aqueous solution of 40% methanol and 10% acetic acid. The stained cells were then harvested as before, washed once with 0.9% NaCl in aqueous solution, and suspended in 0.5 ml drug exposure medium. A Nikon DIAPHOT-TMD inverted microscope was used to score the cells. Photographs of the stained cells were taken with a 35 mm Nikon SLR camera fitted to the microscope.

Data Analysis. Double-reciprocal plots of initial rates *versus* substrate concentrations were used to estimate all K_m and V_{max} values. Initial rates were determined in duplicate with each of five to eight substrate concentrations to generate each value. Wilkinson weighted linear regression analysis (14) was used to fit lines to the double-reciprocal plot values.

Computer-assisted unweighted regression analysis was carried out using the STATView (Brainpower, Inc., Calabas, CA) statistical program to generate all other straight-line functions.

RESULTS

Preliminary experiments established that NAD(P)-linked aldehyde dehydrogenase-catalyzed oxidation of benzaldehyde was confined to the cytosol, more accurately, the soluble (105,000-g supernatant) fraction of methylcholanthrene-treated (3 μM for 5 days) MCF-7/0 cells, *i.e.*, it was not detected in the particulate (105,000-g pellet) fraction, and that the only detectable aldehyde dehydrogenase present in these cells was ALDH-3. Histochemical experiments established that induction of aldehyde dehydrogenase activity occurred essentially uniformly in MCF-7/0 cells and that the enzyme activity was uniformly lost when methylcholanthrene was removed from the medium (Fig. 1). Comparison of the isoelectric focusing pattern and pI values of a crude preparation of this enzyme (Lubrol-treated MCF-7/MC whole homogenate) with those of authentic type-1 and type-2 ALDH-3s strongly suggested that the MCF-7/MC enzyme was a

Table 1 *Purification of a cytosolic class-3 aldehyde dehydrogenase from human methylcholanthrene-treated MCF-7/0 breast adenocarcinoma cells*[a]

Purification step	Total activity (mIU)	Yield (%)	Total protein (mg)	Specific activity (mIU/mg protein)	Fold-purification
Soluble (105,000 *g* supernatant) fraction	3,817	100	68	56	1
CM-Sepharose CL 6B chromatography	3,714	97	46	81	1.4
Reactive Blue 2-Sepharose CL 6B affinity chromatography	2,366	62	0.55	4,302	77
DEAE-Sephacel chromatography	1,945	51	0.059	32,966	589

[a] A concentrated (5 ml) soluble (105,000 *g* supernatant) fraction obtained from MCF-7/0 cells (1.12×10^8) cultured in the presence of 3 μM methylcholanthrene for 5 days was prepared as described in "Materials and Methods" and was then loaded onto a CM-Sepharose CL 6B column (1.5 × 20 cm) equilibrated with Buffer A. Elution was with 70 ml Buffer A, followed by 125 ml Buffer A supplemented with 700 mM NaCl. CM-Sepharose CL 6B column eluates exhibiting aldehyde dehydrogenase activity were pooled (62.5 ml), concentrated (5 ml) as described in "Materials and Methods," and loaded onto a Reactive Blue 2-Sepharose CL 6B column (1.5 × 20 cm) equilibrated with Buffer A. The loaded column was successively eluted with 95 ml Buffer A, 100 ml Buffer B, 100 ml Buffer B supplemented with 2 mM NAD, and 100 ml Buffer B supplemented with 700 mM NaCl. Reactive Blue 2-Sepharose CL 6B column eluates exhibiting aldehyde dehydrogenase activity were pooled (18 ml), concentrated (2 ml), and loaded onto a DEAE-Sephacel column (1.5 × 20 cm) equilibrated with Buffer B. The loaded column was successively eluted with 150 ml Buffer B and 250 ml Buffer B supplemented with 100 mM NaCl. Benzaldehyde (4 mM) and NAD (1 mM) were used to monitor aldehyde dehydrogenase activity.

type-1 ALDH-3 (data not presented). Validation of this suggestion was pursued in the next series of experiments. Purified enzyme was used for this purpose.

Purification of the methylcholanthrene-induced class-3 aldehyde dehydrogenase enzyme was as described in the legend to Table 1. As judged by isoelectric focusing (Fig. 2), nondenaturing linear gradient PAGE (not shown), and SDS-PAGE (Fig. 3), the methylcholanthrene-induced enzyme had been purified to homogeneity. Specific activity, yield and fold-purification of the apparently pure enzyme preparation were 32,966 mIU/mg protein, 51%, and 589 respectively (Table 1). The specific activity of the purified methylcholanthrene-induced enzyme was identical to that of purified stomach mucosa type-1 ALDH-3 (32,951 mIU/mg protein) and substantially different from that of purified MCF-7/OAP type-2 ALDH-3 (16,667 mIU/mg protein) (3).

The methylcholanthrene-induced enzyme exhibited several physical characteristics that were identical to those exhibited by stomach mucosa type-1 ALDH-3 and markedly different from those exhibited by MCF-7/OAP type-2 ALDH-3. Included were pI values (5.75, 5.85, 6.0, 6.25, and 6.35; Fig. 2), the relative native molecular weight [109,000 as determined by nondenaturing linear gradient PAGE and 110,000 as determined by Sephacryl S-200 gel permeation column chromatography (data not shown); these values were M_r 110,000 and 108,000, respectively, for stomach mucosa type-1 ALDH-3 and M_r 110,000 and 125,000, respectively, for MCF-7/OAP type-2 ALDH-3

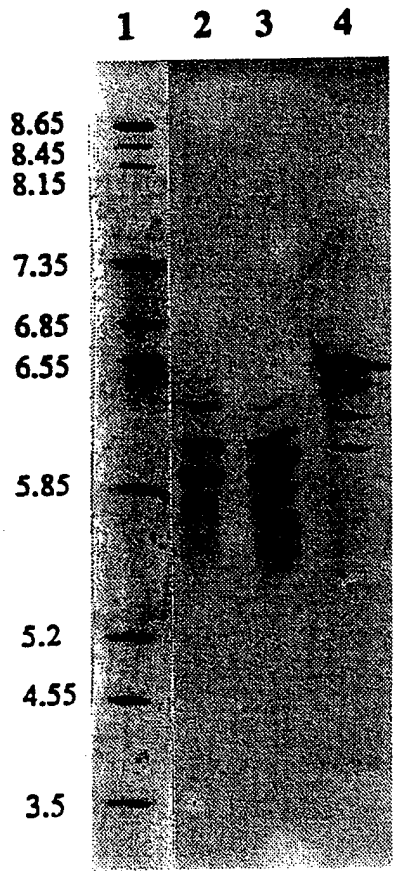

Fig. 2. Isoelectric focusing of the aldehyde dehydrogenase purified from methylcholanthrene-treated MCF-7/0 cells. Induction of ALDH-3 by methylcholanthrene and the subsequent purification of this enzyme were as described in the legend to Table 1. Electrofocusing of pI standards (*Lane 1*) and 5 μg each of the purified methylcholanthrene-induced enzyme (*Lane 3*), purified stomach mucosa type-1 ALDH-3 (*Lane 2*), and purified MCF-7/OAP type-2 ALDH-3 (*Lane 4*) was as described in "Materials and Methods." *Lane 1* was stained with Coomassie Brilliant Blue R-250 for the presence of proteins. *Lanes 2–4* were stained for aldehyde dehydrogenase activity as described in "Materials and Methods"; benzaldehyde (4 mM) and NAD (4 mM) were used as the substrate and cofactor, respectively.

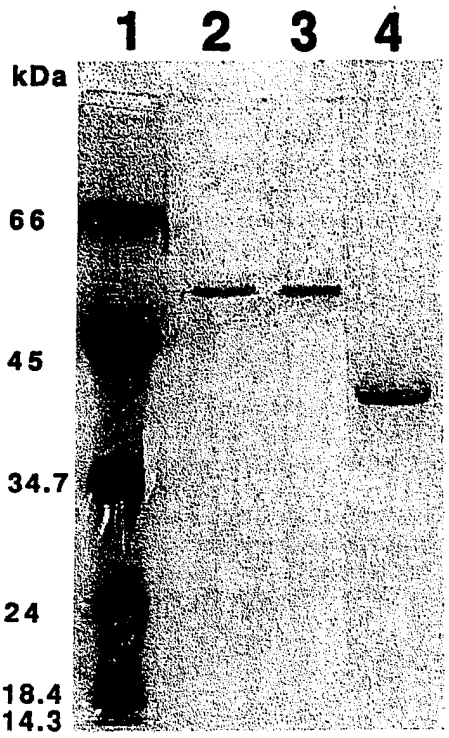

Fig. 3. Subunit molecular weight of the aldehyde dehydrogenase purified from methylcholanthrene-treated MCF-7/0 cells as determined by SDS-PAGE. Induction of ALDH-3 by methylcholanthrene and the subsequent purification of this enzyme were as described in the legend to Table 1. Electrophoresis of molecular weight markers (*Lane 1*), 5 μg of the purified methylcholanthrene-induced enzyme (*Lane 3*), 5 μg of purified stomach mucosa type-1 ALDH-3 (*Lane 2*), and 10 μg of purified MCF-7/OAP type-2 ALDH-3 (*Lane 4*) was as described in "Materials and Methods." Molecular weight markers were lysozyme (14.3 kDa), β-lactoglobulin (18.4 kDa), trypsinogen (24 kDa), pepsin (34.7 kDa), ovalbumin (45 kDa), and BSA monomer (66 kDa). Proteins in each lane were visualized by staining with Coomassie Brilliant Blue R-250. A plot of log M_r *versus* mobility was used as described in "Materials and Methods" to estimate the subunit molecular weight of the methylcholanthrene-induced enzyme.

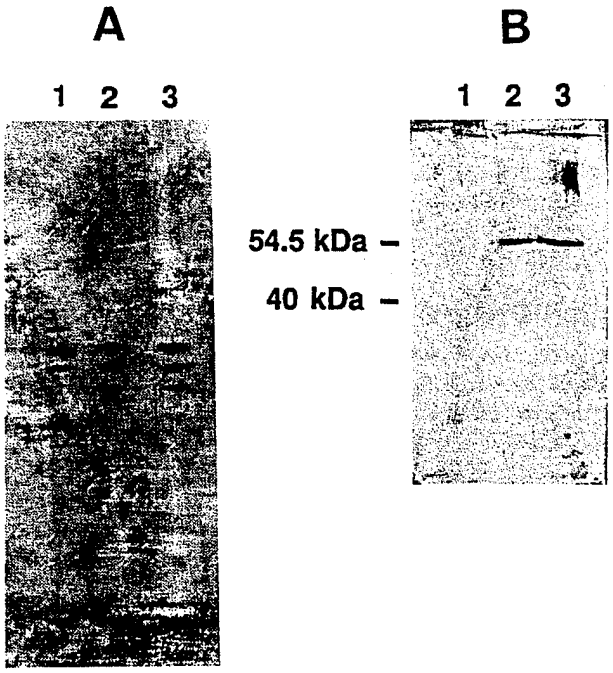

Fig. 4. Immunoblot visualization of native and denatured aldehyde dehydrogenase purified from methylcholanthrene-treated MCF-7/0 cells and submitted to isoelectric focusing (native) or SDS-PAGE (denatured). Induction of ALDH-3 by methylcholanthrene and the subsequent purification of this enzyme were as described in the legend to Table 1. Anti-stomach mucosa type-1 ALDH-3 IgY was generated and used as described in "Materials and Methods" to visualize purified MCF-7/OAP type-2 ALDH-3 (*Lane 1*), the purified methylcholanthrene-induced enzyme (*Lane 2*), and purified stomach mucosa type-1 ALDH-3 (*Lane 3*) after these enzymes had been submitted to (*A*) isoelectric focusing (nondenaturing conditions) or (*B*) SDS-PAGE (denaturing conditions), and electrotransferred onto Immobilon-PVDF transfer membranes. Placed on gels were 5 μg of each purified enzyme, except in the case of the MCF-7/OAP type-2 ALDH-3 submitted to SDS-PAGE where 10 μg of the purified enzyme was placed on the gel.

(3)], the relative subunit molecular weight (M_r 54,500; Fig. 3), and recognition of both the native and denatured enzyme by anti-stomach mucosa type-1 ALDH-3 IgY (Fig. 4).

The ability of the purified methylcholanthrene-induced enzyme to catalyze the oxidation of benzaldehyde was completely lost within 20–25 days when it was kept at 4°C in Buffer B (pH 7.5) ±10% glycerol, whereas full catalytic activity was retained for at least 4 months when it was placed in Buffer B (pH 7.5) and stored frozen at –20°C. Freezing and thawing of the methylcholanthrene-induced enzyme more than two times caused complete loss of catalytic activity (data not shown). While the storage behavior of the methylcholanthrene-induced enzyme was closer to that exhibited by stomach mucosa type-1 ALDH-3 (3), it was not identical to it.

The substrate preference exhibited by the methylcholanthrene-induced enzyme was consistent with that characteristically exhibited by cytosolic class-3 aldehyde dehydrogenases. Thus, as compared to long-chain aliphatic aldehydes (octanal) and to aromatic aldehydes (benzaldehyde and 4-pyridinecarboxaldehyde), a short-chain aliphatic aldehyde (acetaldehyde) was a poor substrate (Table 2). K_m and V_{max} values defining the catalysis of the oxidation of the above-mentioned substrates to their respective acids by the methylcholanthrene-induced enzyme were not unlike those obtained with stomach mucosa type-1 ALDH-3 (3). However, the methylcholanthrene-induced enzyme catalyzed the oxidation of aldophosphamide to carboxyphosphamide, albeit seemingly poorly, whereas stomach mucosa type-1 ALDH-3 essentially did not (3); K_m and V_{max} values were similar to those obtained with MCF-7/OAP type-2 ALDH-3, which also catalyzes the reaction (3) and, as was the case with MCF-7/OAP type-2 ALDH-3, the reaction did not proceed when NADP rather than NAD was used as the cofactor.

NAD and NADP could each serve as the cofactor for the methylcholanthrene-induced enzyme when the substrate was other than aldophosphamide, *e.g.*, benzaldehyde, but, as judged by K_m values, the preferred cofactor was NAD (Table 2). The K_m values reported herein for NAD and NADP (catalysis of benzaldehyde oxidation by the methylcholanthrene-induced enzyme) were virtually identical to those obtained when catalysis of benzaldehyde oxidation by stomach mucosa type-1 ALDH-3 was measured (3). In contrast, the K_m value of 55 μM obtained in the present investigation for NAD was markedly different from the K_m value of 550 μM for NAD obtained previously when catalysis of benzaldehyde by MCF-7/OAP type-2 ALDH-3 was measured (3). As in the case of stomach mucosa type-1 ALDH-3 (3), cofactor "activation" was observed in the case of methylcholanthrene-induced enzyme-catalyzed oxidation of benzaldehyde when the concentration of NAD exceeded 1.0 mM (data not shown); in contrast, cofactor inhibition was observed in the case of MCF-7/OAP type-2 ALDH-3-catalyzed oxidation of benzaldehyde when these concentrations of NAD were used (3). High concentrations of NADP did not inhibit or activate the methylcholanthrene-induced enzyme (data not shown) or inhibit or activate stomach mucosa type-1 ALDH-3 or MCF-7/OAP type-2 ALDH-3 (3).

Like stomach mucosa type-1 ALDH-3 and MCF-7/OAP type-2 ALDH-3, the methylcholanthrene-induced enzyme exhibited esterolytic activity (8,750 mIU/mg protein). Values of 9,800 and 3,350 mIU/mg protein were obtained previously for the stomach mucosa type-1 ALDH-3 and MCF-7/OAP type-2 ALDH-3, respectively (3). As in the case of stomach mucosa type-1 ALDH-3 and MCF-7/OAP type-2 ALDH-3 (3), catalysis of *p*-nitrophenyl acetate hydrolysis by the methylcholanthrene-induced enzyme was enhanced by 20 μM NAD and partially inhibited by 100 μM NAD (data not shown).

The methylcholanthrene-induced enzyme was, like stomach mucosa type-1 ALDH-3 (3), slightly more heat-labile than MCF-7/OAP type-2 ALDH-3 (3); catalytic activity was completely lost in less than 10 min when the enzyme was incubated at 56°C (data not shown).

The influence of agents known to enhance or inhibit the catalytic activity of various aldehyde dehydrogenases, on the ability of the purified methylcholanthrene-induced enzyme to catalyze the oxidation of benzaldehyde, is summarized in Table 3. The sensitivity of the methylcholanthrene-induced enzyme to these agents is virtually identical to the sensitivity to these agents exhibited by stomach mucosa type-1 ALDH-3 (3).

Table 2 *Class-3 aldehyde dehydrogenase purified from methylcholanthrene-treated MCF-7/0 cells: catalytic constants*[a]

Substrate (mM)	Cofactor (mM)	K_m (μM)	V_{max} (mIU/mg)	V_{max}/K_m (mIU/mg/μM)
Benzaldehyde (0.05–4)	NAD	435	34,498	79
	NADP	455	50,563	111
4-Pyridinecarboxaldehyde (0.01–4)	NAD	143	21,735	152
	NADP	159	35,398	223
Octanal (0.01–1)	NAD	159	19,226	121
	NADP	159	23,460	148
Acetaldehyde (4–200)	NAD	85,000	20,147	0.24
	NADP	88,000	22,619	0.26
Aldophosphamide(hdyrate) (0.16–0.96)	NAD	526	405	0.77
	NADP		0	
Benzaldehyde	NAD (0.02–1)	55	28,610	520
	NADP (0.1–4)	909	69,000	76

[a] Kinetic constants were determined as described in "Materials and Methods." Stock purified enzyme preparation was in Buffer B and was added to the reaction mixture in a volume of 100 μl. NAD and NADP concentrations were 1 and 4 mM, respectively, when K_ms defining the enzyme-catalyzed oxidation of benzaldehyde, 4-pyridinecarboxaldehyde, octanal, acetaldehyde, and aldophosphamide(hydrate) were determined. The benzaldehyde concentration was 4 mM when K_ms defining the enzyme-catalyzed reduction of NAD and NADP were determined. Each value is the mean of 3 determinations.

Table 3 *Influence of various effectors on the catalytic activity of class-3 aldehyde dehydrogenase purified from methylcholanthrene-treated MCF-7/0 cells*[a]

Effector	Cofactor	Concentration of effector (μM)	% control ALDH activity
		25	78
	NAD	50	74
		250	47
Disulfiram			
		25	87
	NADP	50	73
		250	18
		10	87
	NAD	25	8
p-Chloromercuribenzoate			
	NADP	10	94
		25	6
	NAD	50	100
		1000	96
Chloral hydrate			
	NADP	50	100
		1000	98
	NAD	250	100
		500	100
Mg^{2+}			
	NADP	250	100
		500	98

[a] Purified enzyme was transferred from Buffer B into 25 mM sodium phosphate buffer, pH 7.5, with the aid of a PD-10 column prior to assay, and aldehyde dehydrogenase activity was measured as described in "Materials and Methods" except that the complete reaction mixture, minus the substrate, was preincubated at 37°C for 5 min. Benzaldehyde (4 mM) was the substrate, and NAD (1 mM) or NADP (4 mM) was the cofactor. Glutathione was omitted from the reaction mixture when disulfiram, *p*-chloromercuribenzoate, and chloral hydrate were tested; EDTA was left out when Mg^{2+} was tested. Control rates ranged from 10 to 12 (NAD) and 21 to 25 (NADP) mIU/min. Values are the means of triplicate determinations.

Collectively, the foregoing observations/deliberations are overwhelmingly consistent with the notion that the methylcholanthrene-induced enzyme is a type-1 rather than a type-2 ALDH-3. However, the storage properties and catalytic behavior (most notably with regard to catalysis of aldophosphamide oxidation) exhibited by this enzyme are not exactly identical with those exhibited by prototypical stomach mucosa type-1 ALDH-3; thus, it may be prudent to consider it a slight variant thereof.

We have reported previously (5) that MCF-7/0 cells grown for 5 days in medium containing 3 μM methylcholanthrene exhibited not only markedly elevated levels of class-3 aldehyde dehydrogenase activity but also a marked decrease in sensitivity to the cytotoxic action of mafosfamide and that ALDH-3 levels returned to basal levels within 15 days after removal of methylcholanthrene from the incubation medium. We now report that, upon the removal of methylcholanthrene, the sensitivity of these cells to the cytotoxic action of mafosfamide returns to basal levels in a parallel manner (Fig. 5). Thus, the methylcholanthrene-induced acquired resistance to mafosfamide exhibited by MCF-7/MC cells is transient, and the two parameters, class-3 aldehyde dehydrogenase activity and sensitivity to oxazaphosphorines, appear to be inversely related, consistent with the notion that loss of sensitivity to the oxazaphosphorines is effected by increases in ALDH-3 activity.

Additional support for this notion was provided by the experiment presented in Fig. 6. Thus, inclusion of benzaldehyde or octanal, aldehydes that are relatively good substrates for the methylcholanthrene-induced enzyme (Table 2), in the drug exposure medium restored the sensitivity of methylcholanthrene-treated MCF-7/0 cells to the cytotoxic action of mafosfamide, whereas inclusion of acetaldehyde, an aldehyde that is a very poor substrate for the methylcholanthrene-induced enzyme (Table 2), did not. Inclusion of benzaldehyde (5 mM) in the drug exposure medium only very minimally increased the sensitivity of MCF-7/0 cells to mafosfamide (LC_{90}s were 54 and 60 μM in the presence and absence of benzaldehyde, respectively) and did not change the sensitivity of either MCF-7/0 or MCF-7/MC cells to phosphoramide mustard (data not shown).

As in MCF-7/0 cells, very small amounts of aldehyde dehydrogenase activity (NADP-linked catalysis of benzaldehyde oxidation) were found in two additional human breast adenocarcinoma cell lines, MDA-MB-231 and SK-BR-3 (Fig. 7). The sensitivity of these cell lines to mafosfamide was very similar to that of MCF-7/0 cells but, unlike methylcholanthrene-treated (3 μM for 5 days) MCF-7/0 cells, methylcholanthrene-treated (3 μM for 5 days) MDA-MB-231 and SK-BR-3 cells did not become resistant to mafosfamide and did not exhibit elevated levels of aldehyde dehydrogenase activity (Fig. 7). Inducibility of aldehyde dehydrogenase in MCF-7/0 cells was concentration dependent, and concentrations higher than 5 μM were toxic (5). In contrast, methylcholanthrene concentrations as high as 30 μM did not induce aldehyde dehydrogenase activity in MDA-MB-231 and SK-BR-3 cells or affect cell growth (data not shown).

In agreement with the report of Whelan *et al.* (15), the glutathione level in MCF-7/0 cells was 185 nmol/10^7 cells [41 nmol/mg cell lysate supernatant (10,000 g) protein]. Glutathione levels were 195 and 30 nmol/10^7 cells in MDA-MB-231 and SK-BR-3 cells, respectively. The glutathione level was not changed when MCF-7/0 cells were exposed to methylcholanthrene (3 μM) for 5 days.

Polycyclic aromatic hydrocarbons are known to induce at least six "drug metabolizing enzymes," namely, cytochrome P450s IA1 and IA2, glutathione *S*-transferase, NAD(P)H:menadione oxidoreductase (DT-diaphorase), UDP-glucuronosyl transferase, and ALDH-3. Induction is believed to be initiated when these agents, known to be ligands for the Ah receptor, bind to it (16). MCF-7/0 cells treated with

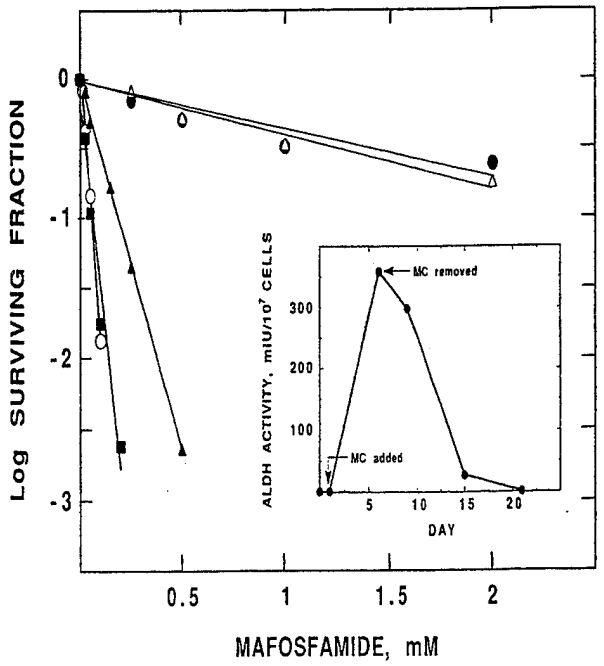

Fig. 5. Effect of removing methylcholanthrene (*MC*) from the culture medium on the sensitivity of methylcholanthrene-treated cells to mafosfamide. Exponentially growing MCF-7/0 cells were cultured in the presence of 3 μM methylcholanthrene for 5 days. At the end of this time, cells were harvested, washed, resuspended in methylcholanthrene-free growth medium, and cultured for an additional 15 days. Sensitivity to mafosfamide was determined as described in "Materials and Methods" on days 0 (○), 6 (●), 9 (△), 15 (▲), and 21 (■). Each point is the mean of measurements on triplicate cultures in each of two separate experiments. LC_{90}s obtained from these plots were 60 (○), >2000 (●), >2000 (△), 190 (▲), and 60 (■) μM. *Inset*, aldehyde dehydrogenase activity at corresponding time points. NADP (4 mM)-linked aldehyde dehydrogenase-catalyzed oxidation of benzaldehyde (4 mM) was quantified in Lubrol-treated whole homogenates as described in "Materials and Methods" at the times indicated. Reaction mixtures (1 ml) contained whole homogenates prepared from 1.5×10^5 to 1×10^7 cells. Each value is the mean of duplicate determinations in each of the two experiments.

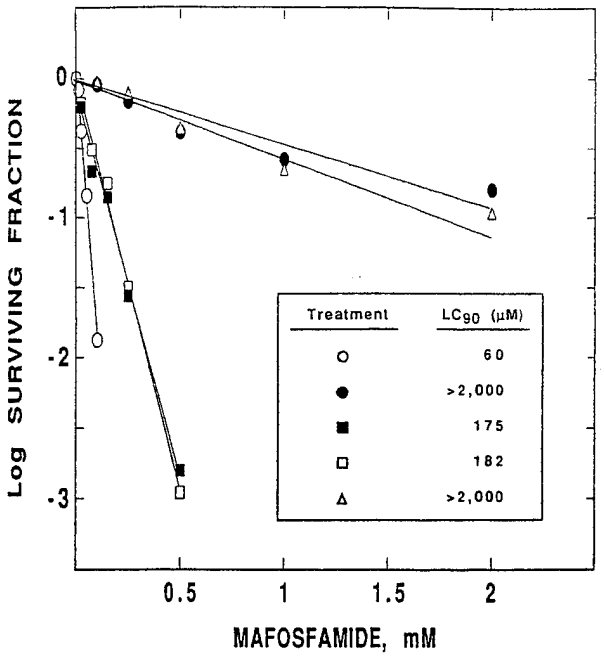

Fig. 6. Sensitivity of methylcholanthrene-treated MCF-7/0 cells to mafosfamide in the presence and absence of benzaldehyde, octanal, and acetaldehyde. Exponentially growing MCF-7/0 cells were cultured in the presence of methylcholanthrene (3 μM) for 5 days and harvested. They were then preincubated at 37°C with 5 mM benzaldehyde (■), 1 mM octanal (□), 5 mM acetaldehyde (△), or vehicle (●) for 5 min; mafosfamide was added, and incubation was continued for an additional 30 min, after which time the cells were harvested, resuspended in drug-free growth medium, and cultured. The colony-forming assay described in "Materials and Methods" was used to determine surviving fractions. Each point is the mean of triplicate cultures in each of two experiments. The sensitivity of untreated MCF-7/0 cells to mafosfamide was also determined and is shown for comparative purposes (○). Aldehyde dehydrogenase activity (4 mM NADP; 4 mM benzaldehyde) in the methylcholanthrene-treated cells was 345 mIU/10^7 cells. LC_{90}s were obtained from these plots and are given in the *inset*.

methylcholanthrene (3 μM for 5 days) also exhibited elevated levels of glutathione *S*-transferase (5-fold) and DT-diaphorase (8-fold) activities; values were 28 and 62, and 150 and 495 mIU/10^7 cells, respectively, in MCF-7/0 and MCF-7/MC cells, respectively. Glutathione *S*-transferase levels in untreated MDA-MB-231 and SK-BR-3 cells were 31 and 565 mIU/10^7 cells, respectively. DT-Diaphorase levels in untreated MDA-MB-231 and SK-BR-3 cells were 59 and 44 mIU/10^7 cells, respectively. Incubation of these cells with 3 μM methylcholanthrene for 5 days did not induce either enzyme activity in either cell line.

Aldehyde dehydrogenase activity was induced in MCF-7/0 cells by each of the three carcinogenic polycyclic aromatic hydrocarbons tested, methylcholanthrene, 3,4-benzpyrene, and 9,10-dimethyl-1,2-benzanthracene (3 μM for 5 days) (5). NADP (4 mM)-dependent aldehyde dehydrogenase-catalyzed oxidation of benzaldehyde (4 mM) was not increased when MCF-7/0 cells were incubated for 5 days with: (*a*) carcinogens that are not polycyclic aromatic hydrocarbons or Ah receptor ligands (2-acetylaminofluorene, diethylnitrosamine, and ethionine; 3 and 300 μM in each case); (*b*) barbiturates known to induce hepatic cytochrome P450s (phenobarbital and barbital; 3 μM and 10 mM in each case); (*c*) steroid hormones (17-β-estradiol, progesterone, androsterone, and corticosterone; 3 and 30 μM in each case); (*d*) an antiestrogen (tamoxifen; 3 and 100 μM); or (*e*) oxazaphosphorines (cyclophosphamide, 3 μM and 1 mM); mafosfamide, 3 and 10 μM; and 4-hydroperoxyifosfamide, 3 and 10 μM). These observations are consistent with those of Huang and Lindahl (17), who reported that aldehyde dehydrogenase activity in rat hepatoma cell lines was induced by several polycyclic aromatic hydrocarbons and by another known Ah receptor ligand, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, but not by 2-acetylaminofluorene, diethylnitrosamine, or ethionine.

Marselos *et al.* (18) reported that treatment of human Hep G2 hepatoma cells with phenobarbital (3 mM) for 2 days followed by methylcholanthrene resulted in the induction of aldehyde dehydrogenase activity that was 2-fold higher as compared to that induced by methylcholanthrene alone. Further elevation of aldehyde dehydrogenase activity was not observed when MCF-7/0 cells were treated with phenobarbital (3 mM for 2 days) followed by methylcholanthrene (3 μM for 5 days).

DISCUSSION

Several conclusions can be made on the basis of the investigational results presented herein. They are: (*a*) the class-3 aldehyde dehydrogenase transiently induced by polycyclic aromatic hydrocarbons in MCF-7/0 cells can be classified as a type-1 ALDH-3, although it may be a slight variant of the prototypical stomach mucosa type-1 ALDH-3; (*b*) the transient oxazaphosphorine-specific acquired resistance exhibited by methylcholanthrene-treated MCF-7/0 cells is accounted for by the transiently elevated levels of type-1 ALDH-3 in these cells but the exact mechanism by which this enzyme brings about the resistance is uncertain; (*c*) the transient induction of type-1 ALDH-3 and the associated oxazaphosphorine-specific acquired resistance effected by methylcholanthrene and all other ligands for the Ah receptor requires the presence, by definition, of Ah receptors in the target cells; and (*d*) transient induction of type-1 ALDH-3 and the associated oxazaphosphorine-specific acquired resistance is not effected by carcinogens that are not ligands for the Ah receptor, barbi-

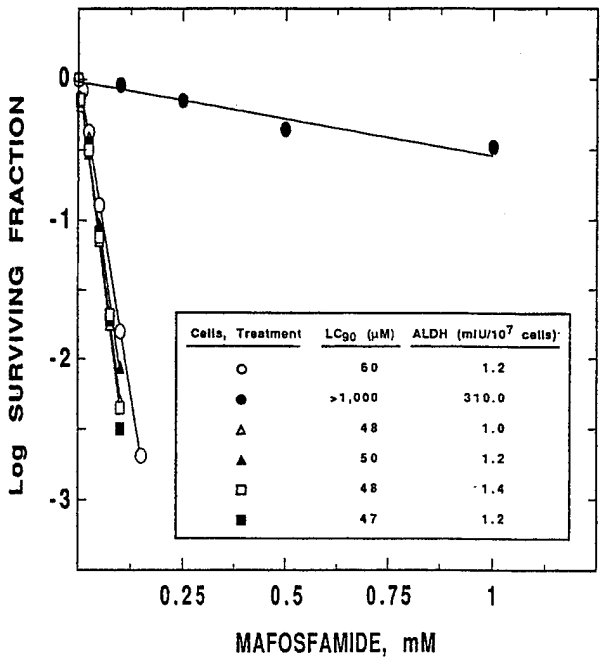

Fig. 7. Sensitivity of human breast adenocarcinoma cells, MCF-7/0, MDA-MB-231, and SK-BR-3, cultured in the presence and absence of methylcholanthrene to mafosfamide. MCF-7/0 (○, ●), MDA-MB-231 (△, ▲), and SK-BR-3 (□, ■) cells were grown in the absence (○, △, □) and presence (●, ▲, ■) of 3 μM methylcholanthrene for 5 days, after which time they were harvested and exposed to mafosfamide for 30 min at 37°C. Following exposure to mafosfamide, cells were again harvested and grown in drug-free growth medium for 15 to 30 days. The colony-forming assay described in "Materials and Methods" was used to determine surviving fractions. Each point is the mean of measurements on triplicate cultures. *Inset*, LC_{90}s and aldehyde dehydrogenase activity. NADP (4 mM)-linked aldehyde dehydrogenase-catalyzed oxidation of benzaldehyde (4 mM) was quantified in Lubrol-treated whole homogenates as described in "Materials and Methods." Reaction mixtures (1 ml) contained whole homogenates prepared from 1.5×10^5 to 1×10^7 cells. Each value is the mean of duplicate determinations.

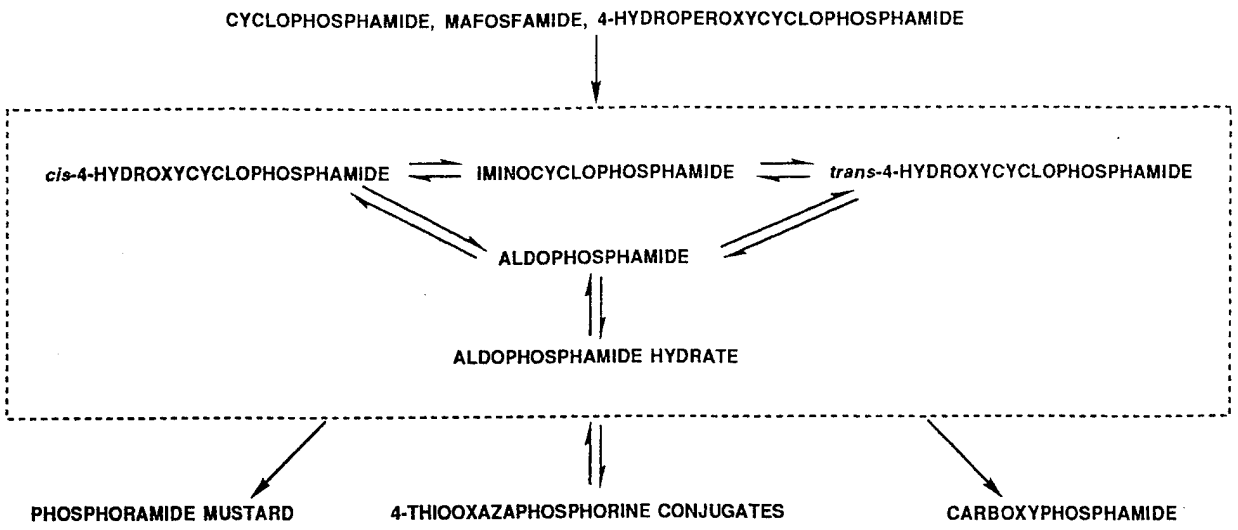

Fig. 8. Some details of oxazaphosphorine metabolism.

turates known to induce hepatic cytochrome P450s, steroid hormones, an antiestrogen, or oxazaphosphorines.

There can be no doubt that the methylcholanthrene-induced class-3 aldehyde dehydrogenase is not a type-2 ALDH-3 and little doubt that it is a type-1 ALDH-3, albeit perhaps a variant thereof since its storage properties and catalytic behavior (most notably with regard to catalysis of aldophosphamide oxidation) are not exactly identical with those exhibited by prototypical stomach mucosa type-1 ALDH-3. This is as expected given that constitutively present in untreated MCF-7/0 cells, as well as in normal breast tissue obtained from pre- and postmenopausal women, are very low levels of type-1 ALDH-3[5] and not type-2 ALDH-3 as we had previously thought (3, 5).

In addition to the evidence presented in "Results" for MCF-7/MC cells and that summarized in the "Introduction" for MCF-7/OAP cells, several other observations support the notion that overexpression of, at least certain, cytosolic class-3 aldehyde dehydrogenases effects oxazaphosphorine-specific resistance.

Not particularly compelling but consistent with the hypothesis is that, in those cases where treatment of tumor cell lines, MDA-MB-231 and SK-BR-3, with methylcholanthrene failed to induce class-3 aldehyde dehydrogenase activity, oxazaphosphorine-specific acquired resistance was also not induced.

As compared to two cultured human colon adenocarcinoma cell lines, RCA and HCT 116b, a third cultured human colon adenocarcinoma cell line, colon C, constitutively expressed much higher (about 200-fold) levels of a cytosolic class-3 aldehyde dehydrogenase and was intrinsically (constitutively) much less sensitive/more resistant to mafosfamide (about 10-fold as judged by LC_{90}s). Resistance was oxazaphosphorine specific. Detectable levels of other aldehyde dehydrogenases were not found in any of the three cell lines. Inclusion of benzaldehyde in the drug exposure medium completely restored the sensitivity of colon C cells to mafosfamide, whereas inclusion of acetaldehyde did not. Inclusion of benzaldehyde in the drug exposure medium only minimally increased the sensitivity of RCA and HCT 116b cells to mafosfamide and did not increase the sensitivity of RCA, HCT 116b, or colon C cells to phosphoramide mustard (19).

Most compelling is the very recent report that in MCF-7/0 cells transfected with a rat cytosolic class-3 aldehyde dehydrogenase complementary DNA, decreased (3- to 4-fold) sensitivity to mafosfamide accompanied increased (20- to 30-fold) expression of the enzyme (20).

Finally, whereas there are several examples showing that cellular sensitivity to, specifically, the oxazaphosphorines decreases (increases) as the cellular expression of cytosolic class-3 aldehyde dehydrogenase increases (decreases), we do not know of any investigations unequivocally showing that increases (decreases) in the cellular expression of cytosolic class-3 aldehyde dehydrogenase is not accompanied by decreases (increases) in cellular sensitivity to, specifically, the oxazaphosphorines.

Assuming that the oxazaphosphorine-specific acquired resistance exhibited by methylcholanthrene-treated MCF-7/0 cells, as well as that exhibited by MCF-7/OAP cells, is indeed the consequence of elevated cytosolic class-3 aldehyde dehydrogenase levels, the question is, how does the latter effect the former? Cytosolic class-3 aldehyde dehydrogenase-catalyzed detoxification (oxidation of aldophosphamide to carboxyphosphamide) immediately comes to mind, but this hypothesis is somewhat problematical since the apparent K_m values defining the catalysis of this reaction by the class-3 aldehyde dehydrogenases expressed in each of these cell populations are quite large [526 (Table 2) and 640 (Ref. 3) μM, respectively], and, in cell-free systems, the reaction proceeds relatively slowly (cytosolic fractions prepared from MCF-7/MC and MCF-7/OAP cells catalyzed the NAD-linked oxidation of aldophosphamide to carboxphosphamide at rates of 2[4] and 2.8 (Ref. 3) nmol/min/10^7 cells, respectively, when the aldophosphamide(hydrate) concentration was 160 μM).

The estimated K_ms may be in error. It is known that the *cis* and *trans* isomers of 4-hydroxycyclophosphamide exist in equilibrium with iminocylophosphamide, aldophosphamide, aldophosphamide hydrate and, when certain thiols are present, 4-thiooxazaphosphorine conjugates (Fig. 8); at full equilibrium, approximately 84% is present as 4-hydroxycyclophosphamide, 4% as aldophosphamide, and 12% as aldophosphamide hydrate when thiols are absent (reviewed in Refs. 2 and 21). What is not known is whether both aldophosphamide and aldophosphamide hydrate are substrates for methylcholanthrene-induced type-1 ALDH-3 (or for type-2 ALDH-3). When calculating K_ms, *e.g.*, 526 μM for methylcholanthrene-induced type-1 ALDH-3, we assumed that both were substrates. In this example, the K_m would be 131 or 395 μM if only aldophosphamide or aldophosphamide hydrate, respectively, was a substrate for this enzyme.

Assuming that methylcholanthrene induced type-1 ALDH-3 is a slight variant of prototypical stomach mucosa type-1 ALDH-3, the

[5] L. Sreerama and N. E. Sladek. Identification of the class-3 aldehyde dehydrogenase present in human MCF-7/0 breast adenocarcinoma cells and normal human breast tissue, submitted for publication.

question arises as to whether relatively elevated levels of the latter would be accompanied by relatively decreased sensitivity to the oxazaphosphorines. The expectation is that it would not if cytosolic class-3 aldehyde dehydrogenase-catalyzed oxidation of aldophosphamide to carboxyphosphamide accounts for the oxazaphosphorine-specific acquired resistance exhibited by MCF-7/OAP and methylcholanthrene-treated MCF-7/0 cells. This is because the prototypical enzyme essentially does not catalyze the reaction; at best, it does so at less than 10% of the rate that the MCF-7/OAP and methylcholanthrene-induced enzymes do.[5] Cytosolic class-3 aldehyde dehydrogenase is constitutively expressed in a number of, though not all, normal tissues in addition to stomach mucosa, *e.g.,* lung, testis, and small intestine mucosa (3, 5). According to the foregoing scenario, the cytosolic class-3 aldehyde dehydrogenase expressed in these tissues is unlikely to protect them from the cytotoxic action of the oxazaphosphorines because, most probably, it is the prototypical enzyme that is expressed in these tissues.

In theory, oxazaphosphorine-specific resistance could be effected by cytosolic class-3 aldehyde dehydrogenase but by a mechanism other than catalysis of aldophosphamide oxidation to carboxyphosphamide. What that mechanism would be is not obvious. A possibility is catalysis of 4-hydroxycyclophosphamide/aldophosphamide hydrolysis to an inactive metabolite since this enzyme also exhibits esterolytic activity when presented with *p*-nitrophenyl acetate.

Even more remote, methylcholanthrene-induced oxazaphosphorine-specific resistance could be effected by a mechanism in which cytosolic class-3 aldehyde dehydrogenase is not a participant. What that mechanism would be is even less obvious given that: (*a*) the methylcholanthrene-induced resistance is largely oxazaphosphorine-specific, and (*b*) inclusion of benzaldehyde or octanal in the drug exposure medium restores the sensitivity of methylcholanthrene-treated MCF-7/0 cells to mafosfamide, whereas inclusion of acetaldehyde does not. Even if not directly related, the two events, overexpression of cytosolic class-3 aldehyde dehydrogenase and decreased cellular sensitivity to the oxazaphosphorines, may be invariably associated.

What is obvious is that quantification of cytosolic class-3 aldehyde dehydrogenase activity in tumor biopsies could be used predictively with regard to the therapeutic potential of oxazaphosphorine chemotherapy, *e.g.,* in breast cancer for which cyclophosphamide is presently the cornerstone of most therapeutic regimens (22–24), if the presence of these enzymes was invariably accompanied, for whatever reason, by decreased cellular sensitivity to these drugs. Quantification of ALDH-1, a known determinant of cellular sensitivity to the oxazaphosphorines (reviewed in Refs. 1, 2, and 21), would also be useful in that regard.

Induction in MCF-7/0 cells of type-1 ALDH-3 activity and the associated oxazaphosphorine-specific resistance, as well as of glutathione *S*-transferase and DT-diaphorase activities, was effected by known ligands for the Ah receptor, *e.g.,* methylcholanthrene, but not by any of several structurally or functionally related agents that are not ligands for this receptor. MCF-7/0 cells express Ah, as well as estrogen and progesterone, receptors (25–27). Methylcholanthrene did not induce the aforementioned enzyme activities or resistance to mafosfamide in two other human breast adenocarcinoma cell lines, MDA-MB-231 and SK-BR-3. MDA-MB-231 cells express Ah but not estrogen or progesterone receptors (25–28). Thus, it may be that, in addition to Ah receptors, estrogen and/or progesterone receptors must be present if induction of type-1 ALDH-3 activity and the associated oxazaphosphorine-specific resistance, as well as of glutathione *S*-transferase and DT-diaphorase activities, is to be effected by Ah receptor ligands. In agreement with this notion, SK-BR-3 cells do not express estrogen receptors (29); it remains to be determined whether they express Ah and/or progesterone receptors. Further supporting this hypothesis is the report that Ah receptor ligand-mediated induction of cytochrome P450 IA1 occurs in several breast tumor cell lines (MCF-7/0, ZR-75B, and T-47D) that express estrogen receptors but not in those (MDA-MB-231 and HS578T) that do not (25, 26). Perhaps relevant, the human estrogen receptor structural gene contains a DNA sequence that binds activated human Ah receptors (30).

Intriguing, too, is that whereas methylcholanthrene inhibits the proliferation of the estrogen receptor-positive MCF-7/0 cells (5), it does not inhibit the proliferation of the estrogen receptor-negative MDA-MB-231 and SK-BR-3 cells. It may be that expression of Ah and estrogen receptors is a requirement for Ah receptor ligand-effected inhibition of cellular proliferation as well, and that inhibition of proliferation is not unrelated to induction of oxazaphosphorine-specific resistance and the various xenobiotic metabolizing enzymes.

Perhaps of paramount significance is that oxazaphosphorine-specific resistance can be not only: (*a*) constitutive and indefinite (colon C); and (*b*) oxazaphosphorine induced and long term/indefinite (MCF-7/OAP); but also (*c*) Ah receptor ligand induced and very short term (MCF-7/MC), although it remains to be demonstrated that the latter ever occurs clinically. The abundance of Ah receptor ligands in the diet/environment (31) would make it seem likely. Exposure to such dietary and environmental ligands, when it occurs, is likely to be continuous, thus oxazaphosphorine-specific resistance is ordinarily not likely to be short term even when insensitivity to the oxazaphosphorines is Ah receptor ligand induced. However, in this scenario, tumor sensitivity to the oxazaphosphorines could be relatively quickly regained by making the appropriate dietary/environmental changes.

While our investigations have thus far focused on oxazaphosphorine-specific resistance, it may be that ligands for the Ah receptor induce, in fact, multidrug resistance and collateral sensitivity as well. As mentioned earlier, Ah receptor ligands induce at least six "drug metabolizing enzymes," namely, cytochrome P450s IA1 and IA2, glutathione S-transferase, DT-diaphorase, UDP-glucuronosyl transferase, and ALDH-3 (16). The levels of at least five of these enzymes, cytochrome P450 IA1, glutathione *S*-transferase, DT-diaphorase, UDP-glucuronosyl transferase, and ALDH-3, have been shown to be elevated in Ah receptor ligand-treated MCF-7/0 cells (see above and Ref. 32). Interestingly, in addition to ALDH-3 activity, those of glutathione *S*-transferase and DT-diaphorase are elevated (approximately 3- and 5-fold, respectively) in MCF-7/OAP cells as well.[4] Thus, the cytotoxic potential of any antitumor agent which is a substrate for these enzymes is likely to be altered when their cellular level is altered. For example, glutathione *S*-transferases catalyze the conjugation of nitrogen mustards such as melphalan and chlorambucil to glutathione, thereby detoxifying these agents (reviewed in Ref. 33). In addition to the large amount of acquired resistance to the oxazaphosphorines exhibited by MCF-7/OAP and methylcholanthrene-treated MCF-7/0 cells, these cells exhibited a small amount of cross-resistance to melphalan and phosphoramide mustard (3, 5). Similarly, UDP-glucuronosyl transferase catalyzes the glucuronidation of mitozantrone and 4-hydroxytamoxifen, thereby almost certainly detoxifying these drugs (34, 35). In contrast, cytochrome P450 IA1 appears to catalyze the bioactivation of ellipticine (reviewed in Ref. 36), and DT-diaphorase apparently catalyzes the aerobic bioreductive activation of a number of compounds including mitomycin C and the indoloquinone, EO9 (reviewed in Refs. 36 and 37). Simultaneous induction of resistance to some agents and collateral sensitivity to others initiated by Ah receptor ligands have obvious relevance with regard to combination therapy, if, in fact, it occurs clinically. Methylcholanthrene-treated MCF-7/0 cells, together with MCF-7/0 cells, may be ideal for the study of potentially useful combinations in this regard.

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Human MCF-7/0 breast adenocarcinoma cells electroporated with essentially equal amounts of class-3 aldehyde dehydrogenase (ALDH-3) obtained from either human normal tissue or tumor cells are rendered differentially resistant to mafosfamide. Sreerama, L. and Sladek, N. E., Department of Pharmacology, University of Minnesota, Minneapolis, MN 55455.

As compared to the ALDH-3 present in human tumor cells, the ALDH-3 present in human normal tissue is apparently much less able to catalyze the oxidation of aldophosphamide [*Biochem. Pharmacol., 48:617-620, 1994*], suggesting that the latter may be much less effective in protecting cells against the cytotoxic action of oxazaphosphorines such as cyclophosphamide and mafosfamide. This notion was tested in the present investigation. Relatively large amounts of human normal stomach mucosa ALDH-3 (nALDH-3) or catechol-induced MCF-7/0 ALDH-3 (tALDH-3) were electroporated into tumor cells, viz., MCF-7/0, that constitutively express only very small amounts of tALDH-3; sensitivity of these preparations to mafosfamide was then determined. Enzyme activities (NADP-dependent catalysis of benzaldehyde oxidation) were 2, 250 and 182 mIU/10^7 cells in sham electroporated MCF-7/0 cells and MCF-7/0 cells electroporated with nALDH-3 and tALDH-3, respectively; LC_{90} values were 62, 340 and >1000 μM, respectively. The three preparations were equisensitive to phosphoramide mustard (LC_{90} = ~850 μM). These observations support the notions that 1) cellular sensitivity to the oxazaphosphorines decreases as cellular content of ALDH-3 increases, 2) the foregoing is the consequence of ALDH-3-catalyzed oxidation (thus inactivation) of aldophosphamide, and 3) tALDH-3 is a slight variant of nALDH-3. Supported by USPHS CA 21737 and DOA DAMD17-94-J-4057.

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HUMAN BREAST ADENOCARCINOMA MCF-7/0 CELLS ELECTROPORATED WITH CYTOSOLIC CLASS 3 ALDEHYDE DEHYDROGENASES OBTAINED FROM TUMOR CELLS AND A NORMAL TISSUE EXHIBIT DIFFERENTIAL SENSITIVITY TO MAFOSFAMIDE

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ABSTRACT:

The cytosolic class 3 aldehyde dehydrogenase (ALDH-3) present in human normal tissues/secretions is apparently much less able to catalyze the oxidation of aldophosphamide to carboxyphosphamide than is the ALDH-3 present in human tumor cells/tissues, suggesting that the former may be less able to protect cells from the cytotoxic action of cyclophosphamide, mafosfamide, and other oxazaphosphorines. To test this notion, relatively large and approximately equal amounts of human normal stomach mucosa ALDH-3 and catechol-induced human breast adenocarcinoma MCF-7/0 ALDH-3 were first electroporated into cells (MCF-7/0) that constitutively express only very small amounts of the enzyme. The resultant preparations were then tested for sensitivity to mafosfamide. ALDH-3 activities (NADP-dependent catalysis of benzaldehyde oxidation) were 1.7, 212, and 183 mIU/10^7 cells in sham-electroporated MCF-7/0 cells, and MCF-7/0 cells electroporated with stomach mucosa ALDH-3 and catechol-induced MCF-7/0 ALDH-3, respectively. LC_{90} values (concentrations of mafosfamide required to effect a 90% cell kill) were 62, 417, and >1,000 μM, respectively. The three preparations were equisensitive to phosphoramide mustard (LC_{90} = ~850 μM). Inclusion of benzaldehyde in the drug exposure medium fully restored the sensitivity of MCF-7/0 cells electroporated with either enzyme to mafosfamide. These observations support the notions that *1)* cellular sensitivity to the oxazaphosphorines decreases as the cellular content of ALDH-3 increases, *2)* the foregoing is the consequence of ALDH-3-catalyzed oxidation (thus detoxification) of aldophosphamide, and *3)* the ALDH-3 present in at least some tumor cells/tissues is a slight variant of the ALDH-3 present in normal tissues/secretions. Furthermore, they illustrate the utility of electroporation used as a tool to determine whether a given enzyme, or even more generally, protein or other macromolecule, is a determinant of cellular sensitivity to a given cytotoxic agent.

ALDH-3[1] is constitutively present in some, but not all, normal and neoplastic tissues/cells (reviewed in refs. 1 and 2). Elevated levels of this enzyme can be induced by certain pharmacological agents, as well as by a wide variety of other xenobiotics (reviewed in refs. 3 and 4), many of which are abundantly present in the environment and/or diet (reviewed in refs. 5–7).

Cellular sensitivity to the widely used antineoplastic drugs collectively known as oxazaphosphorines (*e.g.* cyclophosphamide, 4-hydroperoxycyclophosphamide, and mafosfamide) has been shown to decrease as the cellular content of ALDH-3 is increased (2, 8–14). ALDH-3-effected decreased sensitivity to the oxazaphosphorines is most probably the consequence of increased ALDH-3-catalyzed oxidation of aldophosphamide to carboxyphosphamide, a detoxifying reaction (2, 8–14). Seemingly inconsistent with this notion are the reports that, as judged by cell-free experiments, aldophosphamide is not a very good substrate for this enzyme (8–11, 13, 14).

This study was supported by U.S. Public Health Service Grant CA 21737, Bristol-Myers Squibb Company Grant 100-R220, and DOA Grant DAMD17-94-J-4057. A portion of this study has appeared in abstract form [L. Sreerama and N. E. Sladek, *Proc. Am. Assoc. Cancer Res.* **36,** 325 (1995)].

[1] Abbreviations used are: ALDH-3, cytosolic class 3 aldehyde dehydrogenase; mIU, milli-International Unit of enzyme activity (nmol NADPH formed/min in the case of aldehyde dehydrogenase activity); ALDH, aldehyde dehydrogenase; LC_{90}, drug concentration required to effect a 90% cell kill.

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The ALDH-3 expressed in the two human primary tumors and the two human tumor cell lines thus far examined exhibited physical and catalytic properties that were seemingly identical to those exhibited by the ALDH-3 expressed in the two human normal tissues/secretions thus far examined, except that the latter catalyzed the oxidation of aldophosphamide to carboxyphosphamide at a rate that was, at best, ~10% of that at which did the former (4, 15).

According to this, cells expressing the ALDH-3 found in normal cells should be much more sensitive to the cytotoxic action of oxazaphosphorines than are cells expressing an equal amount of the ALDH-3 found in tumor cells. This notion was tested by first electroporating relatively large and approximately equal amounts of ALDH-3 isolated from human normal stomach mucosa, and ALDH-3 isolated from catechol-treated human breast adenocarcinoma MCF-7/0 cells, into MCF-7/0 cells that otherwise express only very small amounts of ALDH-3, and then quantifying the sensitivity of the resultant preparations to mafosfamide.

Materials and Methods

Mafosfamide was supplied by Dr. J. Pohl (Asta Medica AG, Frankfurt, Germany). Phosphoramide mustard · cyclohexylamine was supplied by the Drug Development Branch, Division of Cancer Treatment, National Cancer Institute (Bethesda, MD). All other chemicals, reagents, and supplies were purchased from commercial sources or prepared as described previously (10, 11).

Human stomach mucosa was provided by the Cooperative Human Tissue Network, Midwest Division (Columbus, OH). Human breast adenocarcinoma MCF-7/0 cells were originally obtained from Dr. B. Teicher, Dana-Farber Cancer Institute (Boston, MA) and were cultured (monolayer) as described

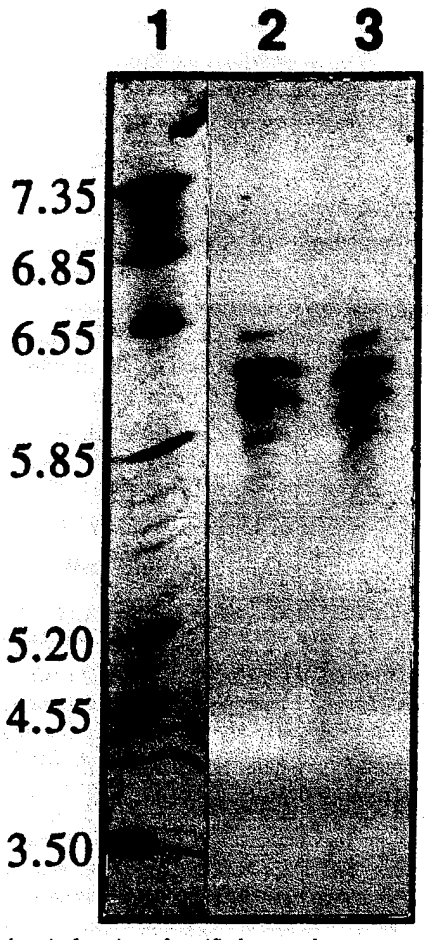

FIG. 1. *Isoelectric focusing of purified stomach mucosa and catechol-induced MCF-7/0 ALDH-3s.*

Purification and isoelectric focusing of ALDH-3s were as described in *Materials and Methods.* Subjected to isoelectric focusing were pI standards (*lane 1*), and amounts of the ALDH-3 purified from stomach mucosa (*lane 2*) and MCF-7/0 cells cultured in the presence of catechol (*lane 3*) sufficient to generate 5 nmol NADH/min (as determined by spectrophotometric assay) when benzaldehyde (4 mM) and NAD (1 mM) were used as substrate and cofactor, respectively. *Lane 1* was stained for the presence of proteins with Coomassie Brilliant Blue R-250. *Lanes 2* and *3* were stained for ALDH activity; benzaldehyde (4 mM) and NAD (4 mM) were used as the substrate and cofactor, respectively.

previously (10, 11). Growth medium was Dulbecco's modified Eagle's medium/10% horse serum supplemented with L-glutamine (2 mM), sodium bicarbonate (3.7 g/liter), and gentamicin (50 mg/liter).

Human stomach mucosa ALDH-3 and catechol-induced MCF-7/0 ALDH-3 were purified from human stomach mucosa and MCF-7/0 cells treated with catechol (30 μM for 5 days) as described previously (10, 14). The physical and catalytic properties of these enzymes were as described previously (10, 14); as in those investigations, each was purified to homogeneity, as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, in the present investigation (data not shown). Isoelectric focusing patterns of the two enzymes are shown in fig. 1. Enzyme storage buffer was 25 mM sodium phosphate (pH 7.5) supplemented with 1 mM EDTA and 0.05% dithiothreitol.

Purified stomach mucosa ALDH-3 and catechol-induced MCF-7/0 ALDH-3 were electroporated into MCF-7/0 cells essentially as described by Potter *et al.* (16). Briefly, MCF-7/0 cells in asynchronous exponential growth (population doubling time ~25 hr) were harvested, resuspended in serum-free growth medium, and checked for viability as described previously (10, 11). The suspended cells were then again harvested, washed 2 times with serum-free growth medium, suspended in serum-free growth medium, and divided into 3 fractions (1–2 × 10^7 cells/fraction). Each of the three fractions was then harvested by low-speed centrifugation (500*g* for 10 min) and resuspended in

TABLE 1

Sensitivity of MCF-7/0 cells electroporated with stomach mucosa ALDH-3 or catechol-induced MCF-7/0 ALDH-3 to mafosfamide[a]

Experiment	Preparation (Electroporation)					
	Sham		Stomach Mucosa ALDH-3		Catechol-Induced MCF-7/0 ALDH-3	
	ALDH-3	LC_{90}	ALDH-3	LC_{90}	ALDH-3	LC_{90}
1	1.60	62	221	453	183	>1,000
2	1.65	62	164	340	184	>1,000
3	1.85	63	250	460	182	>1,000

[a] Values for experiment 1 are from the study presented in fig. 3. Experiments 2 and 3 were conducted in an identical manner, except that the 5-min preincubation with or without benzaldehyde was omitted. Units are mIU/10^7 cells (ALDH-3 activities) and μM (LC_{90} values).

0.9 ml fresh serum-free growth medium. Storage buffer (0.1 ml) was added to one of the fractions. Purified stomach mucosa ALDH-3 [~0.5 mg protein; ~15,000 mIU, benzaldehyde (4 mM) and NAD (1 mM) were substrate and cofactor, respectively] in 0.1 ml of storage buffer was added to a second fraction. Purified catechol-induced MCF-7/0 ALDH-3 [~0.5 mg protein; ~15,000 mIU, benzaldehyde (4 mM) and NAD (1 mM) were substrate and cofactor, respectively] in 0.1 ml storage buffer was added to the third fraction. Each of these preparations was placed in an ice bath for 20 min and then into sterile, disposable, 1-ml cuvettes that had been previously rinsed 3 times with serum-free growth medium. Each preparation was then subjected to an electrical pulse with the aid of an electroporator at 0°–4°C: electrodes were set at a gap of 0.4 cm, voltage was set at 400 V, and the capacitor was set at 25 μF. After electroporation, each of the preparations was placed in an ice bath for 10 min, after which cells were harvested by low-speed centrifugation (500*g* for 10 min) and then washed 3 times with drug exposure medium. Final washings were free of any detectable ALDH activity. An aliquot of each preparation was used to determine the fraction of cells surviving the procedure (~50%) and their viability (>80%). Cells from a second aliquot of each preparation were harvested, resuspended in an aqueous pH 7.4 solution of 1.15% (w/v) KCl and 1 mM EDTA, and sonicated for the purpose of quantifying ALDH activity therein as described previously (10). The remaining cells in each of the preparations were resuspended in drug exposure medium, and their sensitivity to mafosfamide and phosphoramide mustard was determined.

Drug exposure and the colony-forming assay used to determine surviving fractions were as described previously (10, 11). Briefly, the electroporated cells were diluted with drug exposure medium to a concentration of 1 × 10^5 cells/ml and then exposed to drug or vehicle for 30 min at pH 7.4 and 37°C, after which they were harvested and cultured in drug-free growth medium for 20–25 days. Colonies (≥50 cells) were then visualized with methylene blue dye and counted. Plating efficiencies were ~15%.

Isoelectric focusing of purified stomach mucosa, and catechol-induced MCF-7/0, ALDH-3s was as described previously (10). Spectrophotometric quantification of, and histochemical staining assay for, ALDH activity in sham-electroporated MCF-7/0 cells, and MCF-7/0 cells electroporated with ALDH-3, were also as described previously (10, 11).

Computer-assisted unweighted regression analysis was conducted using the STATView (Brainpower, Inc., Calabas, CA) statistical program to generate straight-line functions.

Results

Relatively large quantities of stomach mucosa ALDH-3 and catechol-induced MCF-7/0 ALDH-3 were successfully electroporated into MCF-7/0 cells (table 1, fig. 2). As compared with sham-electroporated MCF-7/0 cells, MCF-7/0 cells electroporated with stomach mucosa ALDH-3 or catechol-induced MCF-7/0 ALDH-3 were substantially less sensitive to mafosfamide (table 1, fig. 3). Moreover, MCF-7/0 cells electroporated with catechol-induced MCF-7/0 ALDH-3 were much less sensitive to mafosfamide than were MCF-7/0 cells electroporated with an approximately equal amount of stomach mucosa

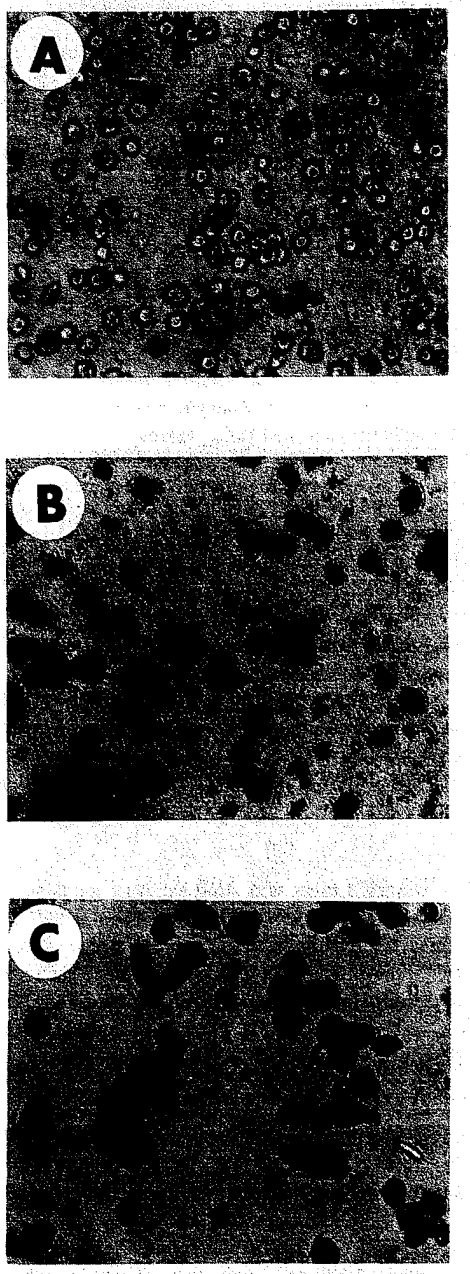

FIG. 2. *Histochemical staining of MCF-7/0 cells electroporated with stomach mucosa ALDH-3 or catechol-induced MCF-7/0 ALDH-3 for ALDH activity.*

Electroporation of stomach mucosa ALDH-3 and catechol-induced MCF-7/0 ALDH-3 into MCF-7/0 cells, and histochemical staining for ALDH activity, were as described in *Materials and Methods.* ALDH activities (4 mM NADP and 4 mM benzaldehyde) in sham-electroporated MCF-7/0 cells (*A*), and in MCF-7/0 cells electroporated with stomach mucosa ALDH-3 (*B*) or catechol-induced MCF-7/0 ALDH-3 (*C*), were, respectively, 1.60, 221, and 183 mIU/10^7 cells.

ALDH-3 (table 1, fig. 3). The differential sensitivity to mafosfamide exhibited by stomach mucosa ALDH-3- and catechol-induced MCF-7/0 ALDH-3-electroporated MCF-7/0 cells was not caused by a difference in the stability of these enzymes after placement into MCF-7/0 cells, because each was equally stable under drug exposure conditions (data not presented). Inclusion of benzaldehyde, a relatively good substrate for ALDH-3s, in the drug exposure medium restored the sensitivity of stomach mucosa ALDH-3- and catechol-induced MCF-7/0 ALDH-3-electroporated MCF-7/0 cells to mafosfamide (fig. 3). As expected, sham-electroporated MCF-7/0 cells and MCF-7/0 cells electroporated with stomach mucosa ALDH-3 or catechol-induced MCF-7/0 ALDH-3 were equisensitive to phosphoramide mustard (LC_{90} = ~850 μM).

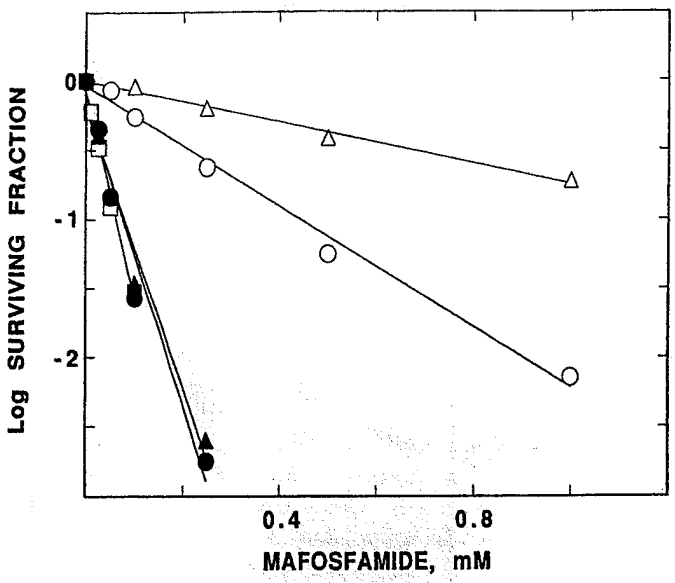

FIG. 3. *Sensitivity of MCF-7/0 cells electroporated with stomach mucosa ALDH-3 or catechol-induced MCF-7/0 ALDH-3 to mafosfamide in the presence of benzaldehyde.*

Electroporation of stomach mucosa ALDH-3 and catechol-induced MCF-7/0 ALDH-3 into MCF-7/0 cells was as described in *Materials and Methods.* MCF-7/0 cells electroporated with stomach mucosa ALDH-3 (○, ●) or catechol-induced MCF-7/0 ALDH-3 (△, ▲) were then preincubated at 37°C with (●, ▲) or without (○, △) 5 mM benzaldehyde for 5 min, mafosfamide was added, and incubation was continued for an additional 30 min, after which time the cells were harvested, resuspended in drug-free growth medium, and cultured. The colony-forming assay described in *Materials and Methods* was used to determine surviving fractions. Each point is the mean value obtained from triplicate cultures. The sensitivity of sham-electroporated MCF-7/0 cells to mafosfamide was also determined and is shown for comparative purposes (□). ALDH activities (4 mM NADP and 4 mM benzaldehyde) in sham-electroporated MCF-7/0 cells, and in MCF-7/0 cells electroporated with stomach mucosa ALDH-3 or catechol-induced MCF-7/0 ALDH-3, were, respectively, 1.60, 221, and 183 mIU/10^7 cells. Concentrations of mafosfamide required to kill 90% of sham-, stomach mucosa ALDH-3-, and catechol-induced MCF-7/0 ALDH-3-electroporated MCF-7/0 cells (*i.e.* LC_{90} values) in the absence of benzaldehyde were, respectively, 62, 453, and >1000 μM. Concentrations of mafosfamide required to kill 90% of stomach mucosa ALDH-3- and catechol-induced MCF-7/0 ALDH-3-electroporated MCF-7/0 cells in the presence of benzaldehyde were, respectively, 70 and 74 μM.

Discussion

Electroporation was originally used to introduce DNA fragments coding for specific genes into cultured mammalian cells (16, 17). Later, it was used to introduce restriction enzymes and various nonpermeable drugs into cultured mammalian cells for the purposes of digesting specific DNA sequences and to study the metabolic fate of these drugs, respectively, within such cells (18–20). Most recently, it was used to introduce a drug-metabolizing enzyme (*viz.* glutathione *S*-transferase π) into mammalian cells for the purpose of establishing a role for this enzyme in catalyzing the detoxification of otherwise cytotoxic α,β-unsaturated aldehydes present in the environment or derived intracellularly (*e.g.* from free-radical-initiated lipid peroxidation) (21). Herein, we again demonstrate that this technology can be used to determine whether a given enzyme, or even more generally, protein or other macromolecule, is a

determinant of cellular sensitivity to a given cytotoxic agent. It offers some obvious advantages over the methodology historically used to make this assessment (*viz.* identification of the operative enzyme after developing a resistant subline, the use of enzyme inducers and inhibitors, and, in some cases, even cDNA transfection).

The present study also establishes unequivocally that there is a causative, and not merely an associative, inverse relationship between cellular content of human ALDH-3, whether of normal or malignant cell origin, and sensitivity to the oxazaphosphorines. Previously, we noted that, in a number of human culture models, invariably associated with an increase in cellular levels of ALDH-3 was a decrease in cellular sensitivity to the oxazaphosphorines (2, 8–11, 13, 14). The fact that *1*) inclusion of relatively good alternative substrates for ALDH-3 in the drug exposure medium abrogated the decreases in cellular sensitivity to the oxazaphosphorines, whereas the inclusion of a poor alternative substrate for this enzyme did not, and 2) the decreases in sensitivity were essentially oxazaphosphorine-specific (*i.e.* did not extend to phosphoramide mustard or other nitrogen mustards), strongly suggested, but did not prove, that ALDH-3 was causative of the decreased sensitivity. Nearly conclusive proof was recently provided by Bunting *et al.* (12). They noted that, upon stable transfection of the cDNA that codes for rat hepatoma class 3 ALDH into MCF-7/0 cells, ALDH-3 levels were modestly elevated and the transfected cells were, as compared with control cells, modestly less sensitive to mafosfamide, 4-hydroperoxycyclophosphamide, and 4-hydroperoxyifosfamide, but equisensitive to phosphoramide mustard and melphalan. In addition to the present study, that described by Bunting and Townsend (22) at the very recently held 86th Annual Meeting of the American Association for Cancer Research provides unequivocal evidence, generated in another way, that elevated levels of ALDH-3 of normal human cell origin are causative of decreased cell sensitivity to the oxazaphosphorines. They found increased cellular levels of ALDH-3 in, and a decreased cellular sensitivity to mafosfamide on the part of, human lung carcinoma V79 (SD1) cells successfully transfected with the cDNA that codes for human normal cell ALDH-3.

Less certain is the catalytic mechanism by which ALDH-3s effect the detoxification of oxazaphosphorines. Oxidation of aldophosphamide to carboxyphosphamide, a detoxifying reaction (8, 9), is, by far, the most attractive possibility. At odds with this notion, however, are the reports that, as judged by cell-free experiments, aldophosphamide is not a very good substrate for ALDH-3, regardless of its source (8–11, 13–15). ALDH-3, like other ALDHs, is a bifunctional enzyme [the two catalytic domains probably overlap (see ref. 23 and references cited therein)] in that it catalyzes the oxidation of aldehydes as well as the hydrolysis of esters (summarized in refs. 4 and 15). Although, there is no evidence to show that it does so, the possibility that ALDH-3 catalyzes the detoxification of oxazaphosphorines by hydrolysis (*e.g.* the phosphoester bond and/or the phosphoramide bond in the cyclic portion of 4-hydroperoxycyclophosphamide) is yet to be ruled out. Arguing against this possibility, but not conclusively refuting it, is the fact that, whereas MCF-7/0 cells electroporated with human normal stomach mucosa ALDH-3 were substantially more sensitive to mafosfamide than were MCF-7/0 cells electroporated with essentially equal amounts of catechol-induced human breast adenocarcinoma MCF-7/0 ALDH-3, the two enzyme preparations were essentially identically able to catalyze the hydrolysis of *p*-nitrophenyl acetate to *p*-nitrophenol (reviewed in ref. 4). In an attempt to resolve this issue, we expect to develop methodology that will allow us to identify and quantify the metabolites that are generated when aldophosphamide is incubated with ALDH-3.

Of particular potential clinical significance (*e.g.* diagnostically) are the present and previous observations (4, 15) suggesting that, at least some, neoplastic cells express a tumor-specific subtle variant of the class 3 ALDH expressed in normal cells. An alternative possibility is that the variant enzyme is tissue-specific. These possibilities are currently under exploration in our laboratory.

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OVEREXPRESSION OF GLUTATHIONE S-TRANSFERASES, DT-DIAPHORASE AND AN APPARENTLY TUMOR-SPECIFIC CYTOSOLIC CLASS-3 ALDEHYDE DEHYDROGENASE BY WARTHIN TUMORS AND MUCOEPIDERMOID CARCINOMAS OF THE PAROTID GLAND[a]

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Running title: ALDH-3 in normal salivary glands and salivary gland tumors.

Key words: Aldehyde dehydrogenase, glutathione S-transferase, DT-diaphorase, salivary glands, parotid glands, salivary gland tumors, Warthin tumors, mucoepidermoid carcinomas, cyclophosphamide, aldophosphamide, oxazaphosphorines.

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FOOTNOTES

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[c]Abbreviations: ALDH-3, cytosolic class-3 aldehyde dehydrogenase; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; pI, isoelectric point; mIU, milli-International Unit of enzyme activity (nmol NAD(P)H formed/min in the case of aldehyde dehydrogenase activity, nmol of the conjugate of 1-chloro-2,4-dinitrobenzene and glutathione formed/min in the case of glutathione S-transferase activity, nmol of 2,6-dichlorophenol-indophenol reduced/min in the case of DT-diaphorase activity, and nmol *p*-nitrophenol formed/min in the case of esterase activity).

SUMMARY

Cytosolic class-3 aldehyde dehydrogenase (ALDH-3) may aid in protecting organisms from certain environmental aldehydes by catalyzing their detoxification. Consistent with this notion are the reports that relatively high levels of this enzyme are present in tissues, e.g., stomach mucosa and lung, that are so-called "ports of entry" for such agents. Further, it is found in human saliva. The present investigation revealed that small amounts of this enzyme are also present in human salivary glands; mean values for ALDH-3 activities (NADP-dependent enzyme-catalyzed oxidation of benzaldehyde) in cytosolic fractions prepared from submandibular and parotid glands were 52 (range: 29 - 92) and 44 (range: 13 - 73) mIU/g tissue, respectively. Essentially identical or slightly lower levels of this enzyme activity were found in pleomorphic adenomas, an undifferentiated carcinoma, and an adenocystic carcinoma, of the parotid gland. On the other hand, Warthin tumors, and mucoepidermoid carcinomas, of the parotid gland exhibited relatively elevated levels of ALDH-3 activity; mean values were 1,200 (range: 780 - 1,880) and 810 (range: 580 - 1,200) mIU/g tissue, respectively. The ALDH-3 found in normal salivary glands was, as judged by physical, immunological and kinetic criteria, identical to human stomach mucosa ALDH-3 whereas the ALDH-3 present in Warthin tumors, and mucoepidermoid carcinomas, of the parotid gland appeared to be a subtle variant thereof. Qualitatively paralleling the relatively elevated ALDH-3 levels in mucoepidermoid carcinomas and Warthin tumors were relatively elevated levels of glutathione S-transferase (α and π) and DT-diaphorase levels. As was the case with ALDH-3, glutathione S-transferase (α and π) and DT-diaphorase levels were not elevated in pleomorphic adenomas. Glutathione S-transferase μ was not detected in the two normal parotid gland samples, or in the single pleomorphic adenoma sample, tested. It was found in the single mucoepidermoid carcinoma sample, and in one of the two Warthin tumor samples, tested. Cellular levels of ALDH-3, glutathione S-transferases and/or DT-diaphorase could be a useful criteria when the decision to be made is whether a salivary gland tumor is a mucoepidermoid carcinoma. ALDH-3 and glutathione S-transferases are known to catalyze the detoxification of two agents that are used to treat salivary gland tumors, viz., cyclophosphamide and cisplatin, respectively. Thus elevated levels of these enzymes in the mucoepidermoid carcinomas must account for, or at least contribute to, the relative ineffectiveness of these agents when used to treat this tumor.

INTRODUCTION

Cytosolic class-3 aldehyde dehydrogenase (ALDH-3)[c] is currently the subject of extensive investigation by our laboratory because it is a demonstrated determinant of cellular sensitivity to certain widely used anticancer drugs, viz., cyclophosphamide, ifosfamide, 4-hydroperoxycyclophosphamide and mafosfamide (cellular sensitivity to these agents decreases as the cellular concentration of ALDH-3 increases) [Sladek, 1993; 1994; Sreerama and Sladek, 1993a,b; 1994a; Bunting, Lindahl and Townsend, 1994; Rekha, Sreerama and Sladek, 1994; Sreerama, Rekha and Sladek, 1995a]. ALDH-3, like other aldehyde dehydrogenases, is a bifunctional enzyme in that it catalyzes the oxidation of aldehydes as well as the hydrolysis of esters [Sreerama and Sladek, 1994b; Sladek, Sreerama and Rekha, 1995]. Hepatic levels of this enzyme are ordinarily very low or even nil, but substantial amounts of it are found in certain other cells, especially those that line the alimentary canal; thus, high levels of this enzyme are found in stomach mucosa, and lower, but still substantial, levels are found in the small and large intestinal mucosa [Sreerama and Sladek, 1993a,b, and references cited therein]. It is also found in human saliva [Sreerama, Hedge and Sladek, 1995b]. Additionally, it is present at high levels in several tumor cell lines that exhibit intrinsic or acquired resistance to mafosfamide and other oxazaphosphorines [Sreerama and Sladek, 1993b; 1994a; Rekha et al., 1994; Sreerama et al., 1995a]. Interestingly, the enzyme found in tumor cells appears to be a slight variant of the one found in normal cells [Sreerama and Sladek, 1994b; Sreerama and Sladek, 1995].

Endogenous substrates for ALDH-3 have yet to be identified and its biological role is uncertain if not unknown. Its *raison d'etre* may be to detoxify xenobiotics. Additionally or alternatively, its purpose may be to catalyze the detoxification of otherwise toxic aldehydes arising from lipid peroxidation thereby protecting cells in which the enzyme is present and such aldehydes arise [reviewed in Lindahl, 1992].

Cellular levels of ALDH-3 and certain other enzymes, viz., glutathione S-transferases and DT-diaphorase, can be coordinately induced by various environmental contaminants, e.g., 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (dioxin, TCDD) and polycyclic aromatic hydrocarbons such as methylcholanthrene, [Lindahl, 1992, Sreerama and Sladek, 1993b, 1994a], as well as by many dietary constituents, e.g., phenolic antioxidants such as catechol and *tert*-butylhydroquinone [Sreerama et al., 1995a].

Recently, we reported the presence of ALDH-3, glutathione S-transferases α, μ and π, and DT-diaphorase in human saliva, and that salivary levels of these enzymes were elevated

in individuals consuming relatively large quantities of coffee and broccoli [Sreerama et al., 1995b]. The origin of salivary ALDH-3, glutathione S-transferases α, μ and π, and DT-diaphorase was not unequivocally established, but a likely possibility is the salivary glands since glutathione S-transferases α, μ and π are known to be present in normal salivary glands [Corrigall and Kirsch, 1988; Campbell et al., 1991; Zeiper et al., 1994]. Glutathione S-transferase π is also reported to be present in salivary gland tumors [Campbell et al., 1991; Zeiper et al., 1994]. Further supporting this notion, preliminary investigations in our laboratory revealed the presence of ALDH-3 activity in normal salivary glands as well as in salivary gland tumors. Of interest, also, was that cellular levels of this activity were sometimes highly elevated in certain salivary gland tumors. The results of these and additional investigations directed towards the quantification of ALDH-3, as well as glutathione S-transferase and DT-diaphorase, activities in normal and neoplastic salivary gland tissue are reported herein. Also, reported is additional evidence supporting the notion that the ALDH-3 present in tumor cells is a slight variant of the one found in normal cells.

MATERIALS AND METHODS

4-Hydroperoxycyclophosphamide was supplied by Dr. J. Pohl, Asta-Medica AG, Frankfurt, Germany. Purified human glutathione S-transferases α, μ and π and affinity-purified polyclonal antibodies specific for each of these isozymes [Townsend et al., 1989] were provided by Dr. A. J. Townsend, Department of Biochemistry, Bowman Gray School of Medicine, Wake Forest University, Winston-Salem, NC. All other chemicals, reagents and supplies were purchased from commercial sources or prepared as described previously [Sreerama and Sladek, 1993a, 1994a; Sreerama et. al., 1995b].

Human normal stomach mucosa, human normal salivary glands (submandibular and parotid) and human salivary gland tumors were obtained from the Cooperative Human Tissue Network, Midwestern Division, Columbus, OH.

Purification of ALDH-3s from various sources and preparation of chicken anti-stomach ALDH-3 IgY were according to protocols described previously [Sreerama and Sladek, 1993a; Sreerama et. al., 1995b].

Soluble (105,000 g supernatant) fractions of normal salivary glands and salivary gland tumors were prepared as described previously for lung and placenta [Sreerama and Sladek, 1993a] except that the homogenization medium was 1.15% (w/v) KCl and 1 mM EDTA in aqueous solution, pH 7.4, and tumors containing excessive connective tissue were minced well in homogenization medium prior to homogenization in a Dounce homogenizer with the aid of an Omni-mixer (Sorvall, model 17105, Norwalk, CT). Soluble (105,000 g supernatant) fractions thus obtained were used as such when aldehyde dehydrogenase, glutathione S-transferase and DT-diaphorase activities present therein were to be quantified. They were further processed when they were to be submitted to SDS-PAGE or isoelectric focusing or when column chromatographic purification of ALDH-3 was to be attempted, viz., they were desalted with the aid of PD-10 (Sephadex G-25) columns (isoelectric focusing, column chromatographic purification) and/or concentrated with the aid of Centricon-10 (Amicon Division. W. R. Grace & Co., Danvers, MA) concentrators by low-speed centrifugation (isoelectric focusing, column chromatographic purification, SDS-PAGE). Microsomal fractions were prepared as described previously [Sreerama et al., 1995a], except that final suspension was in 250 mM sucrose solution when UDP-glucuronosyl transferase activity was quantified.

Spectrophotometric assays for aldehyde dehydrogenase, esterase, glutathione S-transferase and DT-diaphorase activities; determination of protein concentrations; non-denaturing gradient PAGE; SDS-PAGE; isoelectric focusing; protein visualization; and staining for aldehyde dehydrogenase activity were as described previously [Sreerama and Sladek, 1993a, 1994a]. Preliminary experiments with purified enzymes established that ALDH-3s (whether of normal or tumor cell origin), but not ALDH-1, catalyzed NADP-dependent oxidation of benzaldehyde. UDP-glucuronosyl transferase and cytochrome P450 IA1/2 activities were quantified spectrofluorimetrically as described previously [Mackenzie and Hanninen, 1980; Sreerama et al., 1995a]. Electrotransfer and immunoblot analysis of ALDH-3s and glutathione S-transferases were also essentially as described before [Sreerama and Sladek, 1993a; Townsend et al., 1989]; antibody dilutions were 1:500 and 1:1000 in the case of ALDH-3 and glutathione S-transferases, respectively.

Double-reciprocal plots of initial rates *versus* substrate concentrations were used to estimate all Km values. Initial rates were determined in duplicate for each of the 6 to 8 substrate concentrations used to generate each value. Wilkinson weighted linear regression analysis [Wilkinson, 1961] was used to fit the lines to the double-reciprocal plot values.

Computer-assisted unweighted regression analysis was carried out using the STATView (Brainpower, Inc., Calabas, CA) statistical program to generate all other linear functions.

RESULTS

Initial studies revealed that soluble (105,000 g supernatant) fractions prepared from normal submandibular and parotid glands catalyzed NADP-dependent oxidation of benzaldehyde to benzoic acid, Table 1, as well as NAD-dependent oxidation of acetaldehyde to acetic acid (data not presented), albeit at low rates. NADP-dependent enzyme-catalyzed oxidation of benzaldehyde to benzoic acid was always much higher in certain parotid gland neoplasms, viz., Warthin tumors and mucoepidermoid carcinomas, Table 1. It was not higher in certain other neoplasms, viz., pleomorphic adenomas, an undifferentiated carcinoma and an adenocystic carcinoma. Enzyme activity was essentially confined to the 105,000 g supernatant fractions, i.e., only negligible amounts of these activities were present in Lubrol-solubilized particulate (105,000 g pellet) fractions (data not presented).

Isoelectric focusing of the proteins present in soluble (105,000 g supernatant) fractions of the above samples followed by staining for aldehyde dehydrogenase activity (cofactor was NAD; substrates were benzaldehyde, octanal and acetaldehyde), revealed banding patterns characteristic of two aldehyde dehydrogenases, viz., ALDH-3 (2-4 bands within the pI range 5.7-6.4) and ALDH-1 (pI 5.2) (data not shown); in all cases ALDH-3 was, relative to ALDH-1, present in great excess (>15-fold). In the case of Warthin tumors, 2-3 bands were present within the pI range 5.7 - 6.4. In the case of normal tissues as well as all other neoplastic tissues, 4 bands were seen within this pI range. Similar experiments with Lubrol-solubilized 105,000 g pellet fractions obtained from the above-mentioned normal and neoplastic salivary gland tissues revealed the presence of a single aldehyde dehydrogenase, viz., ALDH-2 (pI 4.9) (data not shown).

Also present in 105,000 g supernatant fractions obtained from normal salivary glands were easily measurable levels of glutathione S-transferase and DT-diaphorase activities, Table 2. Qualitatively paralleling the elevated level of ALDH-3 activity in Warthin tumors and mucoepidermoid carcinomas were elevated levels of glutathione S-transferase and DT-diaphorase activities. Glutathione S-transferase and DT-diaphorase activities, like ALDH-3 activity, were not elevated in a pleomorphic adenoma of the parotid gland.

Constitutively elevated levels of ALDH-3, glutathione S-transferase, DT-diaphorase, as well as of UDP-glucuronosyl transferase and cytochrome P450 IA1/2, activities in Warthin tumors or mucoepidermoid carcinomas would be consistent with the notion of stable upregulation of a signaling/regulatory pathway governing the expression of all these

enzymes, viz., regulation via Ah receptors/xenobiotic responsive elements [Sladek et al., 1995, and references cited therein]. On the other hand constitutively elevated levels of all but cytochrome P450 IA1/2 and, perhaps, UDP-glucuronosyl transferase, activities would be consistent with the notion of stable upregulation of a signaling/regulatory pathway, viz., regulation via antioxidant responsive elements, governing the expression of ALDH-3, glutathione S-transferases, DT-diaphorase and, perhaps, UDP-glucuronosyl transferase, but not cytochrome P450s IA1/2 [Sladek et al., 1995, and references cited therein]. We did not detect any UDP-glucuronosyl transferase or cytochrome P450 IA1/2 activities in microsomal fractions prepared from homogenates of either normal parotid glands or Warthin tumors and mucoepidermoid carcinomas of parotid glands (data not shown). However, the tumor samples had been stored at -70°C for >8 months and microsomal enzymes are known to be unstable on prolonged storage at this temperature [reviewed in Okey, 1990].

As judged by immunoblot analysis, glutathione S-transferases α and π were present in each of the two normal parotid glands examined and the level of each was elevated in the mucoepidermoid carcinoma and the two Warthin tumors, but not in the pleomorphic adenoma, tested, Figure 1. In contrast, glutathione S-transferase μ was not detected in either of the two normal glands, or in the pleomorphic adenoma. It was found in the mucoepidermoid carcinoma and in one of the two Warthin tumors. Shown in this figure, too, is that, as judged by immunoblot analysis also, ALDH-3 is present at relatively elevated levels in the Warthin tumors and mucoepidermoid carcinoma tested, whereas the ALDH-3 level in a pleomorphic adenoma is not elevated, when the comparison is with levels of this enzyme in normal parotid glands.

Evidence to support or refute the notion that the ALDH-3 present in tumor tissues is a variant of that present in normal tissues was sought next. Purified enzymes were needed for this purpose. Purification was achieved as described in Materials and Methods. These procedures have been used by us previously to successfully separate ALDH-3 from other aldehyde dehydrogenases and to obtain apparently pure ALDH-3 from various sources [Sreerama and Sladek, 1993a; 1994a,b; Rekha et al., 1994; Sreerama et al., 1995a,b]. Specific activities of the apparently pure aldehyde dehydrogenases isolated from normal parotid glands, and Warthin tumors and mucoepidermoid carcinomas of parotid glands, are given in Table 3.

The physical (a native molecular weight of 110,000 as judged by non-denaturing linear gradient PAGE, data not presented; a subunit molecular weight of 54,500, Figure 2;

recognition of the denatured enzyme by anti-stomach mucosa ALDH-3 IgY, Figure 3) and catalytic (substrate and cofactor preferences as judged by Km values, Tables 4 and 5; esterolytic activity, Table 6) properties of the ALDH-3s purified from normal parotid glands, and Warthin tumors and mucoepidermoid carcinomas of parotid glands, were essentially identical to those reported previously for stomach mucosa and salivary ALDH-3s and for ALDH-3s purified from human breast adenocarcinoma MCF-7, and human colon C carcinoma, cells [Sreerama and Sladek, 1993a; 1994a; Rekha et al., 1994; Sreerama et al., 1995a,b].

Although the banding patterns obtained on subjecting ALDH-3s purified from normal parotid glands, and Warthin tumors and mucoepidermoid carcinomas of parotid glands, to isoelectric focusing were not exactly identical to the banding patterns obtained with stomach mucosa or salivary ALDH-3s, Figure 4, or with human breast adenocarcinoma MCF-7, or human colon C carcinoma, cells [Sreerama and Sladek, 1993a; 1994a; Rekha et al., 1994; Sreerama et al., 1995b], in that the number and/or relative staining intensity of the bands differed somewhat, they were all characteristic of class-3 aldehyde dehydrogenases in that the pI values were all in the range 5.7 - 6.4; it is well known that isoelectric focusing patterns of class-3 aldehyde dehydrogenases, whether of normal, or tumor, cell origin, vary somewhat with the tissue of origin in that as few as two, and as many as five, bands can be present, and/or that the relative amounts of each band can vary, but the pI values always fall in the range 5.7-6.4 [reviewed in Goedde and Agarwal, 1990; Sreerama and Sladek, 1993a].

Like all other ALDH-3s [Sreerama and Sladek, 1993a; 1994a; Rekha et al., 1994; Sreerama et al., 1995a,b], each of the ALDH-3s under current investigation were only partially (<30%) inhibited by a high concentration of disulfiram (50 μM) and were heat labile, i.e., the catalytic activities of each of the preparations were completely lost within 10 min of incubation at 56°C (data not presented).

As compared to ALDH-3s isolated from tumor cells, viz., human breast adenocarcinoma MCF-7 and human colon C carcinoma cells, ALDH-3s isolated from normal cells/fluids, viz., human stomach mucosa and human saliva, catalyze the oxidation of aldophosphamide to carboxyphosphamide only poorly [Sreerama and Sladek, 1993a; 1994a,b; Rekha et al., 1994; Sladek et al., 1995; Sreerama et al., 1995a,b]. When the ability of the enzyme to catalyze this reaction was normalized by the ability of the same enzyme to catalyze the oxidation of benzaldehyde to benzoic acid, the ALDH-3 isolated from normal parotid glands behaved as did ALDH-3s isolated from other human normal

cells/fluids, viz., stomach mucosa and saliva, whereas ALDH-3s isolated from Warthin tumors and mucoepidermoid carcinomas behaved as did those isolated from other human tumor cells, viz., MCF-7 and colon C cells, Table 7.

DISCUSSION

ALDH-3, glutathione S-transferases and DT-diaphorase are found in human saliva [Sreerama et al., 1995b]. The origin of these enzymes is not known with absolute certainty, but the investigative findings reported herein provide further support for the notion that it is the salivary glands.

Relative to those in normal parotid glands, ALDH-3, glutathione S-transferase and DT-diaphorase levels were always elevated in Warthin tumors and mucoepidermoid carcinomas, but were never elevated in pleomorphic adenomas, originating therefrom, nor were they elevated in an undifferentiated carcinoma or an adenocystic carcinoma originating in this gland. According to the taxonomic classification proposed by Dardick and Burford-Mason [1993], most human salivary gland tumors can be placed into one of four groups, viz., the neoplastic counterparts of 1) luminal and basal/myoepithelial cells; stroma of basal lamina and glycosaminoglycans absent, 2) luminal and basal/myoepithelial cells; stroma of basal lamina and glycosaminoglycans present, 3) luminal cells and 4) basal/myoepithelial cells. Parotid gland tumors in which high levels of ALDH-3, glutathione S-transferase and DT-diaphorase are found, viz., Warthin tumors and mucoepidermoid carcinomas, are thus classified as Group 1 tumors, and at least two of the parotid gland tumors in which ALDH-3, glutathione S-transferase and DT-diaphorase levels are low, viz., pleomorphic adenomas and the adenocystic carcinoma, can be classified as Group 2 tumors. Thus, it is tempting to speculate that the inability to synthesize and accumulate basal lamina and glycosaminoglycans, and increased levels of ALDH-3, glutathione S-transferases and DT-diaphorase, are interdependent, but how is not obvious.

Mucoepidermoid carcinomas and Warthin tumors originate from parotid gland excretory and striated duct cells, respectively, whereas pleomorphic adenomas and adenocystic carcinomas originate from parotid gland intercalated duct/acinar cells [Batsakis, El-Naggar and Luna, 1992]. Levels of glutathione S-transferase π are relatively high in excretory duct cells, somewhat lower in striated duct cells and still lower in intercalated duct cells; the enzyme was not detected in acinar cells [Zieper et al., 1994]. This suggests that mucoepidermoid carcinomas and Warthin tumors are derived from normal parotid gland cells in which ALDH-3, glutathione S-transferase and DT-diaphorase levels are relatively high, whereas pleomorphic adenomas and adenocystic carcinomas are derived from normal parotid gland cells in which, at best, levels of these enzymes are low.

Classification of salivary gland tumors as mucoepidermoid carcinomas on the basis of presently used histopathological criteria is problematic [Burgess et al., 1993]; establishing that cellular levels of ALDH-3, glutathione S-transferases and/or DT-diaphorase are, or are not, elevated may be of assistance in that regard. Unknown is whether salivary levels of these enzymes mirror those of salivary gland tumors. If they invariably do, measurement of salivary levels of these enzymes could also be of, at least, preliminarily diagnostic value. However, a finding that salivary levels of these enzymes are elevated could be misleading in that regard since such elevations are known to be effected by ingestion of various dietary constituents, e.g., coffee and broccoli [Sreerama et al., 1995b]. Also of diagnostic potential is our finding that the ALDH-3 present in Warthin tumors and mucoepidermoid carcinomas, as well as that present in various other human tumors [Sreerama and Sladek, 1994b; Sladek et al., 1995], appears to be a, albeit subtle, variant of that found in normal tissues/fluids, i.e., it appears to be tumor-specific. Release of such an enzyme by the salivary gland tumors into the saliva could allow early, noninvasive, detection of such tumors if a suitable assay, e.g., ELISA, could be developed.

In agreement with the reports of others [Corrigall and Kirsch, 1988; Campbell et al., 1991; Zieper et al., 1994], we did not detect the presence of glutathione S-transferase μ in all of the normal and neoplastic salivary gland samples that we examined. This is not surprising since it has been shown that ~50% of the general population lacks the gene that codes for this enzyme [Board, 1981; Seidegard et al., 1988; Comstock et al., 1990; Harada et al., 1992]. Such individuals are thought to be at high risk for developing cancers of larynx, lung, stomach, colon and bladder [Seidegard et al., 1990; Harada et al., 1992; Lafuente et al., 1993; Brockmoller et al., 1994; Katoh et al., 1995; Szarka et al., 1995]. Glutathione S-transferase μ is present in the saliva of some, but not all, individuals [Sreerama et al., 1995b]. It may well be that a total lack of glutathione S-transferase μ in the saliva reflects the deletion of the gene that codes for this enzyme in such subjects. That being the case, the presence or absence of glutathione S-transferase μ in the saliva could be determined, e.g., by ELISA, to noninvasively ascertain whether an individual lacks the relevant gene and is thus at high risk for developing certain cancers.

Standard treatment of salivary gland tumors is surgical removal followed by, in selected cases, radiotherapy. Five-year survival of patients with high-grade salivary gland tumors such as adenocystic carcinomas, adenocarcinomas, mixed malignant tumors and mucoepidermoid carcinomas is only ~50%. In large part, this is because salivary gland tumors often metastasize, e.g., to the lungs, the most favored site [Airoldi et al., 1994, and references cited therein]. Whereas, locoregional recurrences can usually be successfully

managed with additional surgery and/or radiotherapy, more distant metastatic salivary gland tumors cannot, because the location of the metastatic tumors cannot be adequately pinpointed. In such cases, combination chemotherapy is used. Most often used for this purpose are cyclophosphamide, mitomycin C, cisplatin, methotrexate, adriamycin and 5-fluorouracil [Airoldi et al., 1994, and references cited therein]. Combinations of cyclophosphamide, cisplatin and other drugs are of therapeutic value in the treatment of most salivary gland tumors, but such combinations are of limited value in the treatment of mucoepidermoid carcinomas [Rentschler et al., 1977; Creagan et al., 1983; Dimery et al., 1990; Airoldi et al., 1994]. Almost certainly contributing to the lack of sensitivity to cyclophosphamide and cisplatin on the part of mucoepidermoid carcinomas are the high levels in these tumors of ALDH-3 and glutathione S-transferases, as these enzymes catalyze the detoxification of cyclophosphamide and cisplatin, respectively [reviewed in Tsuchida and Sato, 1992; Sladek, 1993; Sreerama and Sladek, 1994a; Sreerama et al., 1995b]. That being the case, addition of agents to the chemotherapeutic protocol that inhibit these enzymes should serve to sensitize these tumor to cyclophosphamide and cisplatin.

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Table 1. Enzyme activity (NADP-dependent catalysis of benzaldehyde oxidation) in 105,000 g supernatant fractions prepared from normal and neoplastic salivary glands[a]

Salivary gland	Microscopic diagnosis	n	Enzyme Activity (mIU/g tissue)	
			Range	Mean ± SD
Submandibular	Normal	6	29 - 92	52 ± 24
Parotid	Normal	7	13 - 73	44 ± 22
	Pleomorphic adenoma[b]	6	3 - 70	45 ± 22
	Warthin tumor[b]	13	730 - 1,880	1,200 ± 370
	Undifferentiated carcinoma	1	-	12
	Adenocystic carcinoma	1	-	24
	Mucoepidermoid carcinoma	6	580 - 1,200	810 ± 220

[a]Benzaldehyde (4 mM) and NADP (4 mM) were used as the substrate and cofactor, respectively, to quantify enzyme activity.

[b]Pleomorphic adenomas and Warthin tumors are benign tumors; all other tumors are malignant.

Table 2. ALDH-3, glutathione S-transferase and DT-diaphorase activities in 105,000 g supernatant fractions prepared from normal and neoplastic salivary gland tissues[a]

Salivary gland	Microscopic diagnosis	Enzyme Activity (mIU/g tissue)		
		ALDH-3	Glutathione S-Transferase	DT-Diaphorase
Submandibular	Normal	48	2,680	580
Parotid	Normal	68	3,100	520
	Normal	54	3,200	740
	Pleomorphic adenoma	50	2,200	790
	Warthin tumor	1,450	14,200	2,300
	Warthin tumor	1,680	16,600	4,100
	Mucoepidermoid carcinoma	1,200	10,500	1,500

[a]Preparation of normal and neoplastic salivary gland soluble (105,000 g supernatant) fractions and determination of ALDH-3 (NADP-dependent catalysis of benzaldehyde oxidation), glutathione S-transferase and DT-diaphorase activities were as described in Materials and Methods. Each value is the mean of three determinations on each tissue sample.

Table 3. ALDH-3s purified from normal parotid glands, and Warthin tumors and mucoepidermoid carcinomas of parotid glands[a]

Source	Specific Activity (mIU/mg protein)	Yield (%)	Fold-Purification
Normal parotid glands	31,800	80	30,600
Warthin tumors	32,000	55	2,920
Mucoepidermoid carcinomas	32,800	67	5,330

[a]ALDH-3s were purified as described in Materials and Methods. Benzaldehyde (4 mM) and NAD (1 mM) were used as substrate and cofactor, respectively, to monitor aldehyde dehydrogenase activity. The specific activities of the purified human stomach mucosa and salivary ALDH-3s used elsewhere in these investigations were 33,000 and 31,000 mIU/mg under identical assay conditions [Sreerama and Sladek, 1993a; Sreerama et. al., 1995b].

Table 4. Substrate preferences of ALDH-3s purified from normal parotid glands, and Warthin tumors and mucoepidermoid carcinomas of parotid glands

Substrate (mM)	Cofactor[a]	Km (μM)				
		Stomach[b] mucosa	Whole[b] saliva	Parotid glands	Warthin tumors	Mucoepidermoid carcinomas
Benzaldehyde (0.05 - 4)	NAD	505	465	450	469	525
	NADP	486	463	460	462	510
Acetaldehyde (25 - 200)	NAD	80,000	85,000	82,000	78,000	79,000
	NADP	81,000	85,000	83,000	87,000	81,000

[a]NAD and NADP concentrations were 1 and 4 mM, respectively.

[b]Km values for stomach mucosa and salivary ALDH-3s are from previous publications [Sreerama and Sladek, 1993a; Sreerama et. al., 1995b]; they are included here for comparative purposes.

Table 5. Cofactor preferences of ALDH-3s purified from normal parotid glands, and Warthin tumors and mucoepidermoid carcinomas of parotid glands

Substrate (mM)	Cofactor (mM)	Km (μM)				
		Stomach[a] mucosa	Whole[a] saliva	Parotid glands	Warthin tumors	Mucoepidermoid carcinomas
Benzaldehyde (4)	NAD (0.01-1)	54	40	42	38	45
	NADP (0.1-4)	1,000	1,250	1,150	1,100	1,200

[a]Km values for stomach mucosa and salivary ALDH-3s are from previous publications [Sreerama and Sladek, 1993a; Sreerama et. al., 1995b]; they are included here for comparative purposes.

Table 6. Esterase activity of ALDH-3s purified from normal parotid glands, and Warthin tumors and mucoepidermoid carcinomas of parotid glands[a]

Source	Esterase Acetivity (mIU/mg)
Stomach mucosa[b]	9,800
Whole saliva[b]	8,390
Parotid glands	8,250
Warthin tumors	8,000
Mucoepidermoid carcinomas	8,780

[a]The rate at which purified ALDH-3s catalyzed the hydrolysis of *p*-nitrophenyl acetate (500 μM) to *p*-nitrophenol was determined as described in Materials and Methods. Each value is the mean of three determinations.

[b]Values for stomach mucosa and salivary ALDH-3s are from previous publications [Sreerama and Sladek, 1993a; Sreerama et. al., 1995b]; they are included here for comparative purposes.

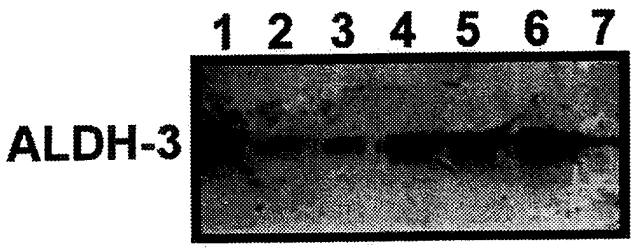
Table 7. Catalysis of aldophosphamide and benzaldehyde oxidation by ALDH-3s purified from various normal and neoplastic tissues/fluids: relative rates[a]

Source of ALDH-3	(nmol Aldophosphamide oxidized/min/mg) (1000) / (nmol Benzaldehyde oxidized/min/mg)
Stomach mucosa[b]	0.29
Whole saliva[b]	0.32
Parotid glands	0.41
Warthin tumors	3.03
Mucoepidermoid carcinomas	3.54

[a]Aldehyde dehydrogenase activity was quantified as described in Materials and Methods; aldophosphamide (160 μM) or benzaldehyde (4 mM) was the substrate and NAD (1 mM) was the cofactor.

[b]Values for stomach mucosa and salivary ALDH-3s are from previous publications [Sreerama and Sladek, 1994b; Sreerama et. al., 1995b]; they are included here for comparative purposes.

Figure 1. *Immunoblot analysis of 105,000 g supernatant fractions prepared from normal parotid glands and several parotid gland tumors for the presence of ALDH-3 and glutathione S-transferases (GST) α, μ and π.* Tissue samples evaluated are those for which global enzyme activity is given in Table 2. One μg each of authentic stomach mucosa ALDH-3 (left panel, lane 1) and glutathione S-transferases α, μ and π (right panels, lane 1), and 105,000 g supernatant fractions (100 μg each) prepared from normal parotid glands (lanes 2 and 3), Warthin tumors of parotid glands (lanes 4 and 5), a mucoepidermoid carcinoma of the parotid gland (lane 6) and a pleomorphic adenoma of the parotid gland (lane 7) were first subjected to SDS-PAGE. Proteins thus resolved were then electrotransferred onto Immobilon-PVDF transfer membranes and probed with antibodies against human ALDH-3 and glutathione S-transferases α, μ and π. Anti-human stomach mucosa ALDH-3 IgY and anti-human liver glutathione S-transferases α, μ and π IgG were prepared and used as described in Materials and Methods.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ALDH-3

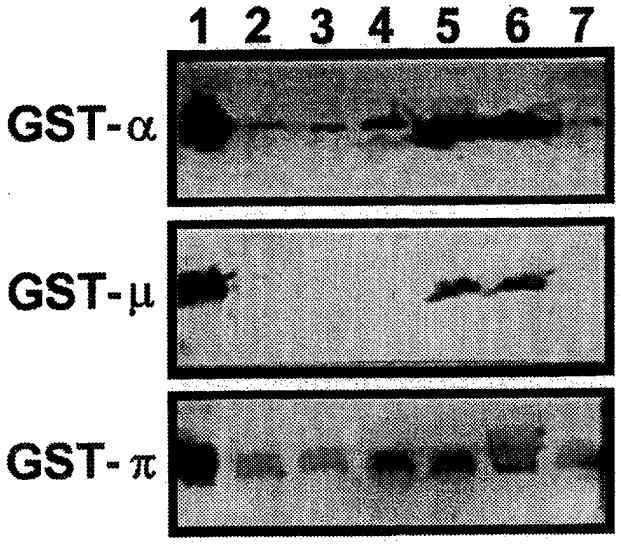

1 2 3 4 5 6 7
GST-α
GST-μ
GST-π

Figure 2. *Subunit molecular weight of ALDH-3s purified from normal parotid glands, and Warthin tumors and mucoepidermoid carcinomas of parotid glands, as determined by SDS-PAGE.* Purification and SDS-PAGE of ALDH-3s were as described Materials and Methods. Subjected to SDS-PAGE were molecular weight markers (lane 1), and 3-5 μg of each of the ALDH-3s purified from stomach mucosa (lane 2), normal parotid glands (lane 3), Warthin tumors of parotid glands (lane 4), mucoepidermoid carcinomas of parotid glands (lane 5) and saliva of a healthy adult human (lane 6). Molecular weight markers were lysozyme (14.3 kDa), trypsin inhibitor (21.5 kDa), carbonic anhydrase (31 kDa), ovalbumin (45 kDa), BSA monomer (66 kDa) and phosphorylase *b* (97.4 kDa). Proteins in each lane were visualized by staining with Coomassie Brilliant Blue R-250. A plot of log M_r *versus* mobility was used to estimate subunit molecular weights.

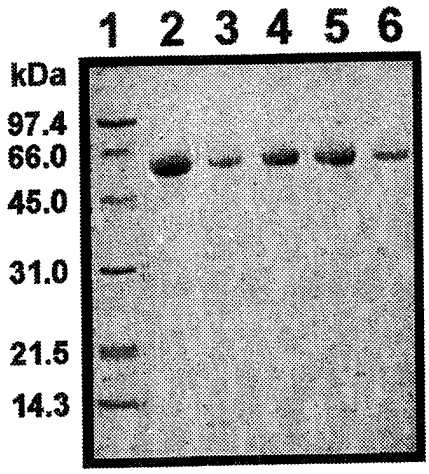

1 2 3 4 5 6
kDa
97.4
66.0
45.0
31.0
21.5
14.3

Figure 3. *Immunoblot visualization of ALDH-3s purified from normal parotid glands, and Warthin tumors and mucoepidermoid carcinomas of parotid glands, and subjected to SDS-PAGE.* Purification and SDS-PAGE of ALDH-3s (1 μg each) were exactly as described in the legend to Figure 2. Electrotransfer of proteins onto an Immobilon PVDF-transfer membrane was as described in Materials and Methods. Anti-stomach mucosa ALDH-3 IgY was generated and used as described in Materials and Methods to visualize ALDH-3s purified from stomach mucosa (lane 1), normal parotid glands (lane 2), Warthin tumors of parotid glands (lane 3), mucoepidermoid carcinomas of parotid glands (lane 4) and saliva of a healthy adult human (lane 5).

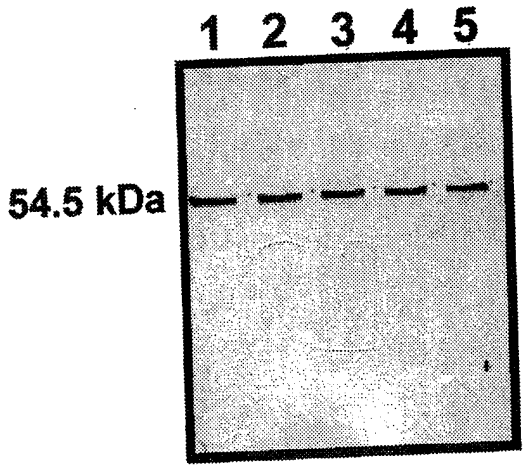
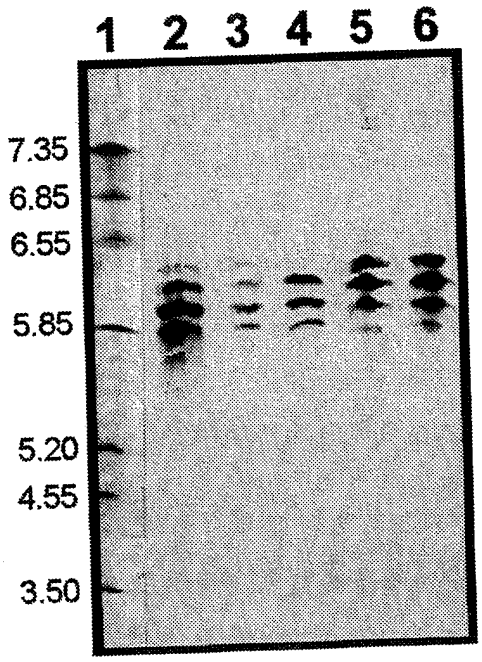

1 2 3 4 5
54.5 kDa

Figure 4. *Isoelectric focusing of ALDH-3s purified from normal parotid glands, and Warthin tumors and mucoepidermoid carcinomas of parotid glands.* Purification and isoelectric focusing of ALDH-3s were as described in Materials and Methods. Subjected to isoelectric focusing were pI standards (lane 1), and amounts of the ALDH-3 purified from stomach mucosa (lane 2), normal parotid glands (lane 3), Warthin tumors of parotid glands (lane 4), mucoepidermoid carcinomas of parotid glands (lane 5) and saliva of a healthy adult human (lane 6), sufficient to generate 5-10 nmol NADH/min (as determined by spectrophotometric assay) when benzaldehyde (4 mM) and NAD (1 mM) were used as substrate and cofactor, respectively. Lane 1 was stained for the presence of proteins with Coomassie Brilliant Blue R-250. Lanes 2-6 were stained for aldehyde dehydrogenase activity; benzaldehyde (4 mM) and NAD (4 mM) were used as the substrate and cofactor, respectively.


1 2 3 4 5 6
7.35
6.85
6.55
5.85
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4.55
3.50

Pergamon

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PHENOLIC ANTIOXIDANT-INDUCED OVEREXPRESSION OF CLASS-3 ALDEHYDE DEHYDROGENASE AND OXAZAPHOSPHORINE-SPECIFIC RESISTANCE

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Abstract—High-level cytosolic class-3 aldehyde dehydrogenase (ALDH-3)-mediated oxazaphosphorine-specific resistance (>35-fold as judged by the concentrations of mafosfamide required to effect a 90% cell-kill) was induced in cultured human breast adenocarcinoma MCF-7/0 cells by growing them in the presence of 30 μM catechol for 5 days. Resistance was transient in that cellular sensitivity to mafosfamide was fully restored after only a few days when the inducing agent was removed from the culture medium. The operative enzyme was identified as a type-1 ALDH-3. Cellular levels of glutathione *S*-transferase and DT-diaphorase activities, but not of cytochrome P450 IA1 activity, were also elevated. Other phenolic antioxidants, e.g. hydroquinone and 2,6-di-*tert*-butyl-4-hydroxytoluene, also induced ALDH-3 activity when MCF-7/0 cells were cultured in their presence. Thus, the increased expression of a type-1 ALDH-3 and the other enzymes induced by these agents was most probably the result of transcriptional activation of the relevant genes via antioxidant responsive elements present in their 5′-flanking regions. Cellular levels of ALDH-3 activity were also increased when a number of other human tumor cell lines, e.g. breast adenocarcinoma MDA-MB-231, breast carcinoma T-47D and colon carcinoma HCT 116b, were cultured in the presence of catechol. These findings should be viewed as greatly expanding the number of recognized environmental and dietary agents that can potentially negatively influence the sensitivity of tumor cells to cyclophosphamide and other oxazaphosphorines.

Key words: aldehyde dehydrogenase; phenolic antioxidants; antioxidant responsive elements; cyclophosphamide; aldophosphamide; mafosfamide; oxazaphosphorines; breast cancer; drug resistance

Phenolic antioxidants, e.g. catechol, BHA† and BHT, are known to induce glutathione *S*-transferase and DT-diaphorase (NAD(P)H:quinone oxidoreductase) activities in various human and rodent organs/tissues/cells [reviewed in Refs. 1 and 2]. Transcriptional activation of the relevant genes by these agents appears to be via ARE present in the 5′-flanking regions of these genes [3–6]. The ARE core sequence required for transcriptional activation of rat glutathione *S*-transferase Ya subunit and DT-diaphorase genes is reportedly 5′-GTGACNNNGC-3′ [4]. This sequence is also found in the 5′-flanking region (−738 to −729) of the rat ALDH-3 gene [7] as well as in that (−462 to −453) of the human DT-diaphorase gene [8, 9]. On the basis of the foregoing, and because cytosolic ALDH-3 mRNA levels are elevated significantly in mouse Hepa 1 cells cultured in the presence of *tert*-butylhydroquinone [10], we reasoned that, since a functional ARE was likely to also be present in the 5′-flanking region of the human ALDH-3 gene, phenolic antioxidants may induce ALDH-3 activity in human organs/tissues/cells, e.g. breast adenocarcinoma MCF-7/0 cells, and, since cellular sensitivity to oxazaphosphorines such as 4-hydroperoxycyclophosphamide and mafosfamide decreases as cellular content of ALDH-3 increases [11–14], phenolic antioxidant-treated cells, e.g. MCF-7/0, may be less sensitive to the oxazaphosphorines than are their untreated counterparts. These expectations were dramatically realized.

MATERIALS AND METHODS

Mafosfamide and phosphoramide mustard·cyclohexylamine were supplied by Dr. J. Pöhl, Asta-Werke AG, Bielefeld, Germany, and the Drug Development Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD, respectively. [γ-^{32}P] ATP and [α-^{32}P] dATP were purchased from New England Nuclear Research Products-Du Pont Co., Boston, MA. T4 polynucleotide kinase

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† Abbreviations: BHA, 3(5)-di-*tert*-butyl-4-hydroxyanisole; BHT, 2,6-di-*tert*-butyl-4-hydroxytoluene; ARE, antioxidant responsive elements; ALDH-3, cytosolic class-3 aldehyde dehydrogenase; XRE, xenobiotic responsive elements; mIU, milli-International Unit of enzyme activity (nmol NAD(P)H formed/min in the case of aldehyde dehydrogenase activity, nmol of the conjugate of 1-chloro-2,4-dinitrobenzene and glutathione formed/min in the case glutathione *S*-transferase activity, nmol of 2,6-dichlorophenol-indophenol reduced/min in the case of DT-diaphorase activity, and nmol of resorufin formed/min in the case of cytochrome P450 IA1 activity); and LC_{90}, drug concentration required to effect a 90% cell-kill.

and a random primed DNA labeling kit were purchased from the USB Corp., Cleveland, OH. An oligonucleotide probe, viz. 5′-ATCCAGCAGCT GGAGGCGCTGCAGCGC-3′, specific (100% homology with cDNA [15]) for the mRNA sequence that codes for the 9 N-terminal end amino acids of stomach mucosa ALDH-3, was synthesized for us by the Microchemical Facility, University of Minnesota Medical School, Minneapolis, MN. 5′-End-labeling of this probe was with T4 polynucleotide kinase and [γ-^{32}P] ATP (3000 Ci/mmol) according to the protocol provided by the USB Corp. Full-length β-actin cDNA was provided by Dr. C. Campbell, Department of Pharmacology, University of Minnesota Medical School, Minneapolis, MN. Random primed labeling of β-actin cDNA was with the random primed labeling kit and [α-^{32}P] dATP (3000 Ci/mmol) according to the protocol provided by the USB Corp. All other chemicals, reagents and supplies were purchased from commercial sources or prepared as before [11, 13].

Purified stomach mucosa type-1 ALDH-3 and MCF-7/OAP type-2 ALDH-3, and anti-stomach mucosa type-1 ALDH-3 IgY, were prepared as described previously [11].

Human breast adenocarcinoma MCF-7/0 and MCF-7/OAP cells were originally obtained from Dr. B. Teicher, Dana-Farber Cancer Institute, Boston, MA. Human breast adenocarcinoma SK-BR-3 cells were provided by Dr. S. Ramakrishnan, Department of Pharmacology, University of Minnesota Medical School, Minneapolis, MN. Human breast adenocarcinoma MDA-MB-231, and human breast carcinoma T-47D and ZR-75-1, cells were purchased from the American Type Culture Collection, Rockville, MD. Human colon carcinoma cells, viz. colon C and HCT 116b, were provided by Dr. M. G. Brattain, Department of Biochemistry and Molecular Biology, Medical School of Ohio, Toledo, OH.

T-47D and ZR-75-1, and colon C and HCT 116b, cells were cultured (monolayer) in, respectively, RPMI-1640 medium/10% fetal bovine serum and Dulbecco's modified Eagle's medium/10% horse serum, each of which was supplemented with L-glutamine (2 mM), sodium bicarbonate (3.7 g/L), basal medium Eagle amino acids solution (6 mL/L), basal medium Eagle vitamin solution (6 mL/L), sodium pyruvate (1 mM), and gentamicin (50 mg/L). Population doubling times were 48, 88, 25 and 22 hr for T-47D, ZR-75-1, colon C and HCT 116b cells, respectively. All other cell lines were cultured at 37° as described previously [11, 13].

Catechol, hydroquinone, *tert*-butylhydroquinone, BHA, BHT, ethoxyquin and Vitamin E were dissolved in dimethyl sulfoxide. The concentration of dimethyl sulfoxide ultimately present in the culture media never exceeded 0.1% and did not affect cell growth or induce aldehyde dehydrogenase, glutathione *S*-transferase, DT-diaphorase or cytochrome P450 IA1 activities.

Lubrol-treated whole homogenates and 105,000 *g* soluble fractions were prepared as described previously [11]. Microsomal fractions were prepared by differential centrifugation. MCF-7/0 cells were suspended in 1.15% KCl (5×10^6–1×10^7 cells/mL) and then were lysed in an ice-bath by submitting them to sonication for a total of 10 sec (divided into 3 bursts). The lysates were subjected to centrifugation at 800 *g* and 4° for 20 min, and the resultant supernatant fractions were further centrifuged at 105,000 *g* and 4° for 60 min. The 105,000 *g* pellets thus obtained (microsomes) were washed once with water and resuspended in 200 μL of water by brief sonication (2 sec). These were the preparations used when cytochrome P450 IA1 activity was to be quantified.

Aldehyde dehydrogenase, glutathione *S*-transferase and DT-diaphorase activities were determined as described previously [13]. Substrates were benzaldehyde (4 mM), 1-chloro-2,4-dinitrobenzene (1 mM) and 2,6-dichlorophenol-indophenol (40 μM), respectively. The method of Burke *et al.* [16] was used to quantify cytochrome P450 IA1 activity. The reaction mixture (1 mL; pH 7.6) contained 100 mM potassium phosphate, 5 μM 7-ethoxyresorufin, 250 μM NADPH and microsomes. The reaction mixture (minus NADPH) was preincubated at 37°

Table 1. ALDH-3, DT-diaphorase, glutathione *S*-transferase and cytochrome P450 IA1 activities in untreated and catechol-treated human MCF-7/0 breast adenocarcinoma cells*

Enzyme	mIU/10^7 cells		Treated/Control
	Control	Treated	
NAD-ALDH-3	1.5 ± 0.1	381 ± 13	254
NADP-ALDH-3	1.7 ± 0.1	768 ± 43	452
DT-Diaphorase	77 ± 4	6395 ± 748	83
Glutathione *S*-transferase	24 ± 2	250 ± 11	10
Cytochrome P450 IA1	0.032 ± 0.003	0.034 ± 0.002	1

* Exponentially growing MCF-7/0 cells (1×10^5) were cultured in the presence of vehicle (control) or 30 μM catechol (treated) for 5 days. They were then harvested, and Lubrol-treated whole homogenates (2.5×10^4–5×10^6 cells) were prepared and assayed for aldehyde dehydrogenase, DT-diaphorase, and glutathione *S*-transferase activities, as described in Materials and Methods. In addition, microsomal fractions (2.5×10^6–1×10^7) were prepared and assayed for cytochrome P450 IA1 activity, as described in Materials and Methods. Each value is the mean ± SEM of four determinations.

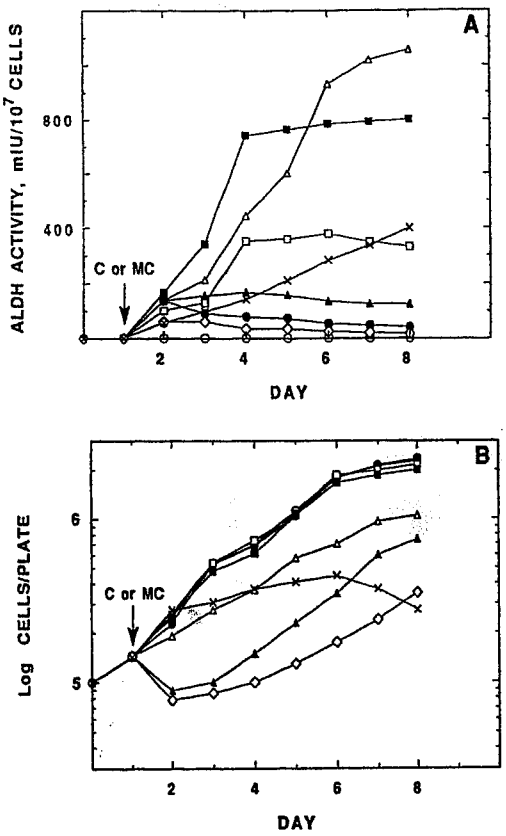

Fig. 1. Induction of aldehyde dehydrogenase (ALDH) activity in MCF-7/0 cells by catechol (C): concentration dependence. Exponentially growing MCF-7/0 cells were exposed continuously to vehicle (○), 3 μM 3-methylcholanthrene (MC) (×), or 3 (●), 10 (□), 30 (■), 60 (△), 100 (▲), or 300 (◇) μM catechol, for 7 days. Lubrol-treated whole homogenates of these cells were then prepared and ALDH activity therein (panel A) was quantified as described in Materials and Methods; benzaldehyde (4 mM) and NADP (4 mM) were used as substrate and cofactor, respectively. Growth of MCF-7/0 cells in the presence of vehicle, 3 μM 3-methylcholanthrene, or various concentrations of catechol is shown in panel B. Values are means of duplicate determinations made in each of two separate experiments.

for 2 min, NADPH was added, and the reaction (O-dealkylation of 7-ethoxyresorufin to resorufin) was monitored with the aid of an automated Spex-3000 spectrofluorimeter; excitation and emission wavelengths were 530 and 585 nm, respectively. Authentic resorufin was used to generate a standard curve. O-Dealkylation of ethyl ethers, e.g. 7-ethoxyresorufin, is preferentially catalyzed by cytochrome P450 IA1 as opposed to cytochrome P450 IA2 [16, 17], and it has been reported that, whereas polycyclic aromatic hydrocarbons induce cytochrome P450 IA1 activity in MCF-7/0 cells, they do not induce cytochrome P450 IA2 activity in these cells [18]. Hence, the assumption is that the O-dealkylation of ethoxyresorufin measured in this investigation was catalyzed by cytochrome P450 IA1.

Chromatographic purification of catechol-induced ALDH-3, protein determinations, SDS–PAGE, immunoblot analysis and data analysis were as described previously [11, 13].

Drug exposure and the colony-forming assay used to determine surviving fractions were as described previously [11–13]. Essentially, freshly harvested cells were diluted with drug-exposure medium to a concentration of 1×10^5 cells/mL and were then exposed to mafosfamide, phosphoramide mustard or vehicle for 30 min at pH 7.4 and 37° after which they were harvested and cultured in drug-free growth medium for 15 days. Colonies (≥50 cells) were then visualized with methylene blue dye and counted.

Isolation of total RNA from cultured cells, isolation of poly(A)$^+$-enriched RNA from total RNA with the aid of oligo(dT)-cellulose columns, resolution of poly(A)$^+$-enriched RNA on agarose gels (1%) containing formaldehyde, transfer of resolved RNA onto Zeta-Probe nylon membranes (Bio-Rad Laboratories, Richmond, CA), hybridization and autoradiographic visualization of the resultant blots were essentially as described by Angelini *et al.* [19] and Sambrook *et al.* [20]. Briefly, Zeta-Probe membranes containing poly(A)$^+$-enriched RNA were baked at 80° for 2 hr and placed in thermoresistant plastic bags. The bags were then filled with 10 mL hybridization solution, pH 7.2, containing 750 mM sodium chloride, 75 mM sodium citrate, 20 mM sodium phosphate, 7% SDS (w/v), 20% Denhardt's reagent (2 mg each of Ficoll, polyvinylpyrrolidone and bovine serum albumin) and 1 mg denatured salmon sperm DNA, sealed and prehybridized for 8 hr at 50°. Freshly prepared 5'-end-labeled ALDH-3 specific oligonucleotide probe was added to the hybridization bag, and incubation was continued overnight (~14 hr). Following hybridization, the membrane was washed twice at 50° for 30 min in a solution, pH 7.2, containing 100 mM sodium chloride, 10 mM sodium citrate, 25 mM sodium phosphate, 5% SDS and 20% Denhardt's reagent, and once at 25° for 30 min in a solution containing 30 mM sodium chloride, 3 mM sodium citrate and 1% SDS. The wet membrane was then wrapped in Saran wrap, and the wrapped membrane, juxtaposed to a Kodak X-OMAT-AR X-ray film, was sandwiched between intensifying screens for 72 hr. The oligonucleotide probe was removed from the membrane by incubating the latter at 95° for 8 hr in a solution containing 3 mM sodium chloride, 0.3 mM sodium citrate and 0.5% SDS. The membrane was then rehybridized with ^{32}P-labeled human β-actin cDNA as described above except that the incubations were at 68°. Films were developed in an automated X-ray film processor (model QX-130A plus, Konica Corp., Japan).

RESULTS AND DISCUSSION

ALDH-3 activity was elevated markedly when human breast adenocarcinoma MCF-7/0 cells were cultured in the presence of 30 μM catechol for 5 days (Table 1). DT-Diaphorase and glutathione *S*-transferase activities were also elevated but to a significantly lesser extent.

Given that the rat ALDH-3 gene, like the rat and human DT-diaphorase and the rat glutathione *S*-

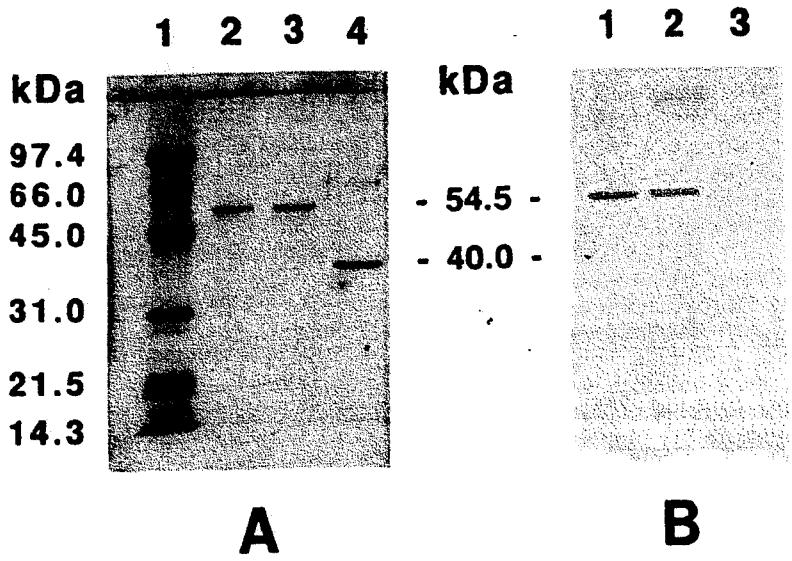

Fig. 2. ALDH-3 purified from catechol-treated MCF-7/0 cells: subunit molecular weight and recognition of the denatured enzyme by anti-stomach mucosa type-1 ALDH-3 IgY. Induction of ALDH-3 by catechol, and the subsequent purification of this enzyme, were as described in Materials and Methods and in Table 1. (Panel A) SDS–PAGE of molecular weight markers (lane 1) and 5 μg each of purified stomach mucosa type-1 ALDH-3 (lane 2), catechol-induced enzyme (lane 3), and MCF-7/OAP type-2 ALDH-3 (lane 4) was as described in Materials and Methods. Molecular weight markers were lysozyme (14.3 kDa), trypsin inhibitor (21.5 kDa), carbonic anhydrase (31 kDa), ovalbumin (45 kDa), BSA monomer (66 kDa) and phosphorylase *b* (97.4 kDa). Proteins in each lane were visualized by staining with Coomassie Brilliant Blue R-250. A plot of log M_r versus mobility was used as described in Materials and Methods to estimate the subunit molecular weight of the catechol-induced enzyme. (Panel B) Purified stomach mucosa type-1 ALDH-3 (lane 1), purified catechol-induced enzyme (lane 2), and purified MCF-7/OAP type-2 ALDH-3 (lane 3) were submitted to SDS–PAGE and electrotransferred onto an Immobilon-PVDF transfer membrane; attempted visualization of the denatured enzymes with anti-stomach mucosa type-1 ALDH-3 IgY was as described in Materials and Methods. Placed on the gel were 5 μg of each purified enzyme.

transferase Ya subunit genes [3–6, 8, 9], contains both XRE and ARE in its 5′-flanking region [7], it is likely that XRE, as well as ARE, are also present in the 5′-flanking region of the human ALDH-3 gene. XRE, but not ARE, are also found in the 5′-flanking region of the cytochrome P450 IA1 gene [reviewed in Refs. 21 and 22]. Induction of cytochrome P450 IA1 activity by 3-methylcholanthrene and other ligands for the Ah receptor is thought to be the result of a sequence of events culminating in the activation of cytochrome P450 IA1 gene transcription when the Ah receptor–ligand complex binds to the XRE present in the 5′-flanking region of this gene [reviewed in Refs. 21 and 22]. Induction of ALDH-3, DT-diaphorase and glutathione *S*-transferase activities by Ah receptor ligands is also thought to be via XRE, but, in addition, (induced) cytochrome P450 IA1 catalyzes the conversion of some of these ligands, e.g. 3-methylcholanthrene, to metabolites that induce the synthesis of these enzymes via ARE [reviewed in Refs. 1 and 2]. Cytochrome P450 IA1 activity was elevated markedly (>10-fold)*, as were ALDH-3, DT-diaphorase, and glutathione *S*-transferase activities [12, 13], when MCF-7/0 cells were cultured in the presence of 3 μM 3-methylcholanthrene for 5 days. Consistent with the notion that catechol induction of ALDH-3, DT-diaphorase and glutathione *S*-transferase activities is directly via ARE, cytochrome P450 IA1 activity was not elevated when MCF-7/0 cells were cultured in the presence of 30 μM catechol for 5 days (Table 1).

NAD(P)-dependent ALDH-3 activity was also elevated markedly when MCF-7/0 cells were cultured in the presence of other phenolic antioxidants for 5 days, viz. 30 μM of either hydroquinone (>150-fold), *tert*-butyl-hydroquinone (>50-fold), BHA (>50-fold), ethoxyquin (>50-fold), BHT (>15-fold), or Vitamin E (~5-fold) (data not shown). Moreover, it was elevated markedly when human breast adenocarcinoma MDA-MB-231 (>500-fold) and SK-BR-3 (~10-fold), human breast carcinoma T-47D (>500-fold) and ZR-75-1 (~5-fold), and human colon carcinoma HCT 116b (>50-fold) and colon C (~4-fold), cells were cultured in the presence of 30 μM catechol for 5 days (data not shown). This distinguishes phenolic antioxidant induction of ALDH-3 expression from that effected by Ah receptor agonists, e.g. polycyclic aromatic hydrocarbons such as 3-methylcholanthrene and 3,4-benzpyrene, since the latter, while inducing ALDH-

* Sreerama L, Rekha GK and Sladek NE, unpublished observations.

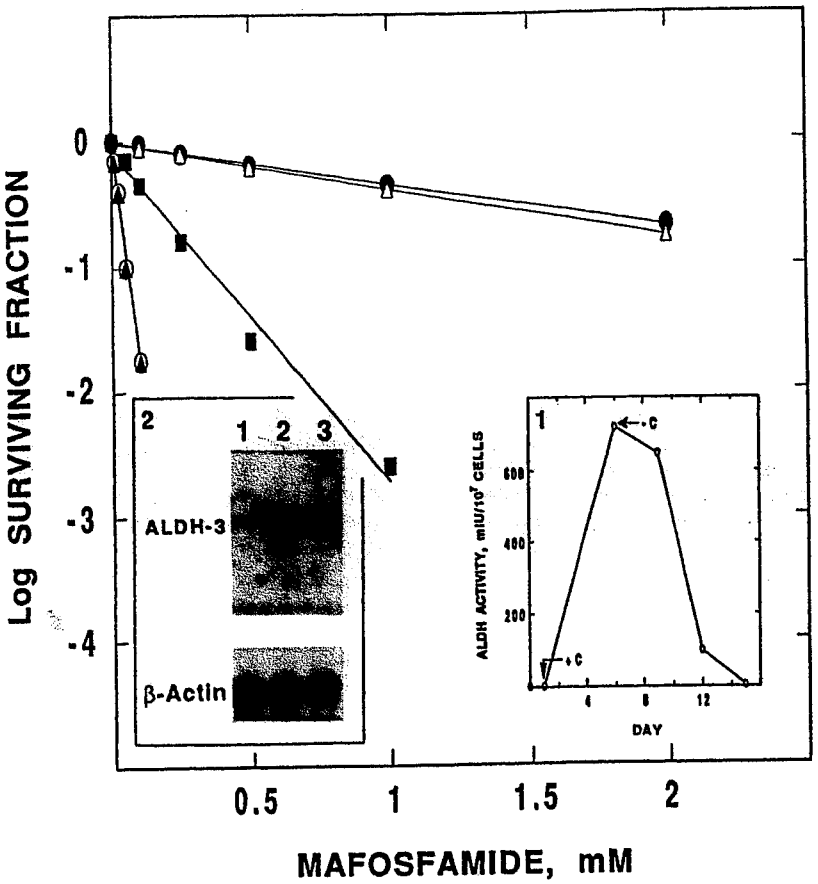

Fig. 3. Effect of adding and then removing catechol from the culture medium on the sensitivity of MCF-7/0 cells to mafosfamide. Exponentially growing MCF-7/0 cells were cultured in the presence of 30 μM catechol (C) for 5 days. At the end of this time, cells were harvested, washed, resuspended in catechol-free growth medium, and cultured for an additional 9 days. Sensitivity to mafosfamide was determined as described in Materials and Methods on days 0 (○), 6 (●), 9 (△), 12 (■), and 15 (▲). Each point is the mean of measurements on triplicate cultures. The LC_{90} values (concentrations of drug required to kill 90% of cells) obtained from these plots were 55 (○), >2000 (●), >2000 (△), 370 (■), and 55 (▲) μM. (Inset 1) Aldehyde dehydrogenase activity at corresponding time points. NADP (4 mM)-linked aldehyde dehydrogenase-catalyzed oxidation of benzaldehyde (4 mM) was quantified in Lubrol-treated whole homogenates, as described in Materials and Methods, at the times indicated. Reaction mixtures (1 mL) contained whole homogenates prepared from 1.5×10^5 to 1×10^7 cells. Each value is the mean of duplicate determinations. (Inset 2) Northern blot analysis of poly(A)$^+$-enriched RNA isolated from MCF-7/0 cells (lane 1), MCF-7/0 cells treated with catechol (30 μM for 5 days) (lane 2), and MCF-7/0 cells treated with catechol (30 μM for 5 days) and then cultured in the absence of catechol for 3 days (lane 3). Isolation of total RNA, isolation of poly(A)$^+$-enriched RNA, electrophoresis of poly(A)$^+$-enriched RNA, transfer of poly(A)$^+$-enriched RNA onto a Zeta-Probe nylon membrane, and probing of the blotted membrane with ^{32}P-labeled oligonucleotide specific for human stomach mucosa type-1 ALDH-3 and with full-length β-actin cDNA were described in Materials and Methods. Placed on the gels were 10 μg of poly(A)$^+$-enriched RNA in each case.

3 expression in MCF-7/0 [13], T-47D*, and ZR-75-1* cells, do not do so in MDA-MB-231, SK-BR-3, HCT 116b, or colon C cells [13, 14].

Induction of ALDH-3 in MCF-7/0 cells by catechol was concentration dependent (Fig. 1). Maximum induction was achieved at a concentration of about 60 μM. A measurable increase in enzyme activity was seen when the catechol concentration was as low as 3 μM. Induction of ALDH-3 activity with the phenolic antioxidant could be achieved without measurably inhibiting cellular proliferation, whereas it could not in the case of Ah receptor agonists [12], thus further distinguishing the two mechanisms of induction.

We have shown previously that ALDH-3s can be of two types, viz. type-1 and type-2 [11]. Type-1 is found in human stomach mucosa, colon C cells, MCF-7/0 cells and 3-methylcholanthrene-treated MCF-7/0 cells [11–14]. Type-2 is found in MCF-7/OAP cells [11]. The latter were generated by growing MCF-7/0 cells in the presence of gradually increasing concentrations of 4-hydroperoxycyclophosphamide

for several months after which time the cells stably expressed high levels of a variant (type-2) ALDH-3 [11, 23]. While the two enzymes share many physical and catalytic characteristics, they also differ in some regards. Thus, the subunit molecular mass of the type-1 enzyme is 54.5 kDa, whereas that of the type-2 enzyme is 40 kDa, and whereas anti-stomach mucosa type-1 ALDH-3 IgY recognizes the type-1 subunit, it does not recognize the type-2 subunit. As judged by these criteria, the ALDH-3 induced by catechol is of the type-1 variety (Fig. 2).

Associated with the marked increase in ALDH-3 expression induced by 30 μM catechol in MCF-7/0 cells was a marked decrease in sensitivity to the cytotoxic action of mafosfamide (Fig. 3). Resistance to mafosfamide was oxazaphosphorine-specific since catechol-treated MCF-7/0 cells were not resistant to phosphoramide mustard; LC_{90} values for untreated and catechol-treated cells were 900 and 1200 μM, respectively (data not presented). Resistance to mafosfamide was transient since sensitivity to mafosfamide, as well as ALDH-3 activity, returned to basal levels within 10 days when catechol was removed from the culture medium. Consistent with transcriptional activation of the ALDH-3 gene by catechol, Northern blot analysis showed that the cellular content of ALDH-3 mRNA was increased markedly when MCF-7/0 cells were grown in the presence of this agent, and that upon its removal, the mRNA level decreased (Fig. 3).

It has now been demonstrated that, in culture models, cytosolic ALDH-3-mediated oxazaphosphorine-specific resistance can be (a) constitutive [14], and (b) induced by exposing cells to gradually increasing concentrations of oxazaphosphorines for several months [11, 23], Ah receptor ligands for a few days [12, 13], or phenolic antioxidants (and, undoubtedly, other ARE activators) for a few days. Constitutive resistance is indefinite by definition. Oxazaphosphorine-induced resistance was long-term, if not indefinite, in the absence of the inducing agent. In contrast, resistance induced by Ah receptor ligands or phenolic antioxidants quickly disappeared when the inducing agent was removed from the culture medium.

The potential significance of "transient" resistance relative to therapeutic strategy is self-evident, but it is yet to be demonstrated that transient resistance to the oxazaphosphorines ever occurs clinically, much less that it is, at least on occasion, the consequence of elevated ALDH-3 levels induced by pharmacological and/or dietary/environmental agents. However, given that only small amounts of ARE activators are needed to induce ALDH-3 expression, and the abundance and widespread distribution of such agents in the diet [reviewed in Refs. 1 and 24], clinical induction of resistance effected by ARE activators would seem especially likely, and an ongoing investigation in our laboratory has yielded preliminary information consistent with this possibility. Especially intriguing is the possibility that, in those cases where resistance to the oxazaphosphorines is induced by ARE activators, sensitivity to these agents could be quickly restored by an appropriate dietary change.

Thus far, our investigations have focused on induction of ALDH-3-mediated oxazaphosphorine-specific resistance. However, it may be that phenolic antioxidants and other ARE activators, as well as Ah receptor ligands, induce, in fact, multidrug resistance and even collateral sensitivity since they "coordinately" induce a number of additional drug-metabolizing enzymes. This notion is more completely discussed in a previous publication [13].

Oxazaphosphorines are also known to be carcinogenic [25]. The cytotoxic and carcinogenic actions of these agents are, at a biochemically fundamental level, almost certainly mechanistically identical. Thus, phenolic antioxidant induction of ALDH-3-mediated resistance to the oxazaphosphorines may also be viewed as a model of cancer chemoprotection. Identification of cancer chemoprotective agents is currently being pursued extensively [26]. In that regard, the determination of ALDH-3, glutathione *S*-transferase and/or DT-diaphorase levels in MCF-7/0 cells, and/or the relative sensitivity of these cells to oxazaphosphorines, e.g. mafosfamide and 4-hydroperoxycyclophosphamide, before and after short-term exposure to candidate agents, may be of value in preliminarily identifying such agents rapidly and economically.

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Identification of a Class 3 Aldehyde Dehydrogenase in Human Saliva and Increased Levels of This Enzyme, Glutathione *S*-Transferases, and DT-Diaphorase in the Saliva of Subjects Who Continually Ingest Large Quantities of Coffee or Broccoli[1]

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ABSTRACT

Human saliva was tested for the presence of cytosolic class 3 aldehyde dehydrogenase, glutathione *S*-transferases α, μ, and π, and DT-diaphorase, enzymes that are known to catalyze the biotransformation of many xenobiotics, including some that are carcinogens and some that are antineoplastic agents. Each of these enzymes was found to be present in this fluid. Inducers of these enzymes are known to be abundantly present in the human diet, especially in certain vegetables and fruits. Further investigation revealed that the salivary content of these enzymes rapidly, coordinately, and markedly increased upon daily consumption of relatively large amounts of coffee or broccoli. The enzyme activities of interest rapidly returned to basal levels when these substances were removed from the diet. Given the important role that cytosolic class 3 aldehyde dehydrogenase, the glutathione *S*-transferases, and DT-diaphorase are thought to play in determining the carcinogenic potential of some cancer-producing agents as well as the cytotoxic potential of some antineoplastic agents, and assuming that their salivary levels reflect their tissue levels, quantification of the salivary content of one or more of these enzymes, a noninvasive and relatively easy undertaking, could be useful in: (*a*) preliminarily assessing the chemopreventive potential of various diets and drugs; (*b*) establishing the optimal dose and schedule in Phase I clinical trials for any putatively chemopreventive diets or drugs of interest; and (*c*) the rational selection and use of chemotherapeutic agents, since several are inactivated, and a few are activated, by these enzymes; alternatively, the antineoplastic agent could be selected first and then a diet that enables the agent to achieve its full therapeutic potential would be selected based on whether high or low enzyme activity would be favorable in that regard. Such measurements may also be useful as an indicator when exposure to carcinogenic/teratogenic/otherwise toxic environmental/industrial/dietary agents that induce these enzymes is suspected.

INTRODUCTION

Cellular expression of ALDH-3[3] and certain other enzymes (*e.g.*, glutathione *S*-transferases and DT-diaphorase) can be markedly and coordinately increased by both monofunctional (*e.g.*, phenolic antioxidants such as catechol, hydroquinone, and 2,6-di-*tert*-butyl-4-hydroxytoluene) and bifunctional (*e.g.*, 3-methylcholanthrene and 3,4-benzpyrene) inducers[4] (1–3). Induction of glutathione *S*-transferases and DT-diaphorase is viewed with particular interest because these enzymes are known to detoxify certain carcinogens (4). Inducers of these enzymes are abundantly present in certain components of the human diet, *e.g.*, members of the *Cruciferae* and *Liliaceae* families of vegetables (5, 6). Certain food additives (*e.g.*, 2,3-*tert*-butyl-4-hydroxyanisole) and pharmaceuticals (*e.g.*, oltipraz) also act as inducers of these enzymes (reviewed in Refs. 7–9). Vegetables, fruits, and chemicals that induce these enzymes prevent experimental carcinogenesis, *i.e.*, they effect a chemopreventive action, and the former is thought to be causative, at least in part, of the latter (reviewed in Refs. 7–9). Chemoprevention mediated in this manner is an immensely attractive idea for obvious reasons, and clinical evaluation of oltipraz in that regard has already been initiated (10–12). Desirable in such investigations is an estimate of relevant enzyme induction. One way of doing this might be to quantify the relevant enzyme activities in serum, peripheral blood lymphocytes, or tissue biopsies (13–15). Limitations of these ap-

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[3] The abbreviations used are: ALDH-3, human cytosolic class 3 aldehyde dehydrogenase; mIU, milli-International Unit of enzyme activity [nmol NAD(P)H formed/min in the case of aldehyde dehydrogenase activity, nmol of the conjugate of 1-chloro-2,4-dinitrobenzene and glutathione formed/min in the case of glutathione *S*-transferase activity, nmol 2,6-dichlorophenol-indophenol reduced/min in the case of DT-diaphorase activity, and nmol *p*-nitrophenol formed/min in the case of esterase activity]; PVDF, polyvinylidene difluoride.

[4] Monofunctional inducers are defined herein as agents that induce ALDH-3, glutathione *S*-transferases, and DT-diaphorase [NAD(P)H: quinone oxidoreductase; NQO_1] but not cytochrome P450s IA1 and IA2; bifunctional inducers are defined herein as agents that induce all of these enzymes (Ref. 28 and references cited therein).

proaches are that each is invasive, the latter two are labor intensive, and the enzyme levels in serum are very low or nil (Ref. 15 and the present study). Another way of doing it would be to determine various pharmacokinetic parameters, *e.g.*, plasma half-life, of an appropriate test substrate before, during, and after exposure to the suspected inducer (16); this approach is also invasive, not likely to be as specific, and, in any event, likely to be even more labor intensive.

It occurred to us that if one or more of the relevant enzymes is present in the saliva, it might be the ideal tissue/fluid to look at. Thus, we searched the literature for reports documenting the presence of at least one of these enzymes in saliva. We were unable to find such a report. However, we did come across an investigation in which the presence of an aldehyde dehydrogenase, apparently different from the known class 1, 2, and 3 aldehyde dehydrogenases and dubbed ALDH-V, in human saliva was demonstrated (17, 18). Although most of the reported catalytic and physical properties of this enzyme did indeed appear to be different from those exhibited by ALDH-3, one, namely, isoelectric point values, was not. Thus, we initiated experiments designed to further evaluate the identity of the salivary aldehyde dehydrogenase and found it to indeed be ALDH-3. In the course of these investigations, we ascertained that, in addition to ALDH-3, glutathione *S*-transferases and DT-diaphorase were also present in the saliva and that levels of each of these enzymes varied widely, but in approximately direct proportion to each other. A review of the gender, age, race, ethnicity, and dietary and other habits of the subjects who had contributed salivary samples strongly pointed to a direct relationship between enzyme activities and coffee consumption. A subsequent review of the literature revealed that coffee contains demonstrated inducers, *e.g.*, catechol, of these enzymes (19). Further experimentation revealed that the continuous consumption of relatively large amounts of coffee did indeed result in markedly increased salivary ALDH-3, glutathione *S*-transferase, and DT-diaphorase levels as did the continuous consumption of relatively large quantities of broccoli, a vegetable known to (*a*) be rich in inducers of these enzymes (5, 6) and (*b*) prevent experimental carcinogenesis (reviewed in Refs. 7, 9, and 20).

MATERIALS AND METHODS

4-Hydroperoxycyclophosphamide was supplied by Dr. J. Pohl (Asta Medica AG, Frankfurt, Germany). Purified human glutathione *S*-transferases α, μ, and π and affinity-purified polyclonal antibodies specific for each of these isozymes (21) were provided by Dr. A. J. Townsend (Department of Biochemistry, Bowman Gray School of Medicine, Wake Forest University, Winston-Salem, NC). Antirabbit IgG alkaline phosphatase conjugate was purchased from Sigma Chemical Co. (St. Louis, MO). Coffee was purchased from the University of Minnesota Food Services Cafeteria (University of Minnesota Medical School, Minneapolis, MN). Broccoli was purchased from a local market, washed, and cooked before consumption. All other chemicals, reagents, and supplies were purchased from commercial sources or prepared as described previously (2, 22).

Human saliva and blood were obtained from healthy adult male and female volunteers ranging from 20 to 55 years of age. Human stomach mucosa was provided by the Cooperative Human Tissue Network (Midwest Division, Columbus, OH). Purified stomach mucosa ALDH-3 and chicken antistomach mucosa ALDH-3 IgY were prepared as described previously (22).

Collection and further processing of saliva was essentially as described by Takase *et al.* (23), except that hyaluronidase was not added to the collected saliva. Briefly, saliva samples (3–5 ml) were collected between 9 and 11 am and were placed into small beakers kept at 4°C. DTT was added (final concentration 5 mM), and the samples were centrifuged at 9000 × *g* and 4°C for 15 min. The supernatant fractions thus obtained were used as such when enzyme activities present therein were to be quantified. They were further processed when they were to be submitted to SDS-PAGE, immunoblot analysis, isoelectric focusing, and/or when column chromatographic purification of the aldehyde dehydrogenase present therein was to be attempted. In those cases, they were desalted with the aid of PD-10 (Sephadex G-25) columns (isoelectric focusing of aldehyde dehydrogenase) and/or concentrated in Centricon-10 concentrators (Amicon Division, W. R. Grace & Co., Danvers, MA) by low-speed centrifugation (isoelectric focusing or column chromatographic purification of aldehyde dehydrogenase; SDS-PAGE and immunoblot analysis of glutathione *S*-transferases). Desalted 9000 × *g* supernatant fractions (obtained from 1.5 ml saliva) were subjected to affinity chromatography on reactive blue 2-Sepharose CL-6B as described previously (22) to eliminate nonspecific proteins, and the resultant preparation was then concentrated as described above when SDS-PAGE/immunoblot analysis for the presence of ALDH-3 was attempted.

Blood (15–20 ml) was collected in heparinized syringes and immediately centrifuged at 2000 × *g* and 4°C for 15 min. Routinely, the plasma thus obtained was then assayed for enzyme activities. In those cases where SDS-PAGE/immunoblot analysis for the presence of ALDH-3 was to be performed, plasma samples were first desalted and subjected to reactive blue 2-Sepharose CL-6B affinity chromatography, after which the resultant fraction of interest was concentrated, all as described above.

The aldehyde dehydrogenase present in the saliva obtained from a single individual ($NADP^+$-dependent enzyme-catalyzed oxidation of benzaldehyde was 41 mIU/ml saliva) was purified by successive DEAE-Sephacel anion exchange chromatography, CM-Sepharose CL-6B cation exchange chromatography, and reactive blue 2-Sepharose CL-6B affinity chromatography as previously described (22), except that elution of aldehyde dehydrogenase off the affinity column was with 1, rather than 5, mM NAD^+.

Electrotransfer and immunoblot analysis of ALDH-3 and glutathione *S*-transferases were essentially as described before (2, 21, 22); antibody dilutions were 1:500 and 1:1000 in the case of ALDH-3 and glutathione *S*-transferases, respectively. All other experimental procedures were as described previously (2, 22).

Double-reciprocal plots of initial rates *versus* substrate concentrations were used to estimate all K_m values. Initial rates were determined in duplicate for each of the six to eight substrate concentrations used to generate each value. Wilkinson weighted linear regression analysis (24) was used to fit lines to the double-reciprocal plot values.

Computer-assisted unweighted regression analysis was carried out using the STATView (Brainpower, Inc., Calabas, CA) statistical program to generate all other linear functions.

Table 1 Aldehyde dehydrogenase purified from human saliva[a]

Measurement	Saliva	Stomach Mucosa[b]
Specific activity (mIU/mg protein)	31,000	33,000
Yield (%)	51	60
Fold purification	1,340	423

[a] Enzyme purification was as described in "Materials and Methods." Benzaldehyde (4 mM) and NAD^+ (1 mM) were used as substrate and cofactor, respectively, to monitor aldehyde dehydrogenase activity. Aldehyde dehydrogenase activity in the starting material (9000 × *g* supernatant fraction of saliva) was 41 mIU/ml.

[b] Values for purified stomach mucosa ALDH-3 are from a previous publication (22); they are included for comparative purposes.

RESULTS

Preliminary studies revealed the presence of an $NAD(P)^+$-dependent enzyme that catalyzed the oxidation of benzaldehyde to benzoic acid in human saliva. The level (concentration) of this enzyme activity in the saliva of any given individual was independent of the time of day at which the sample was collected, or whether the sample was collected just before (10–15 min), just after (10–15 min), or long after (15–16 h) eating. However, it did decrease when the sample volume exceeded approximately 10 ml. Isoelectric focusing of the proteins present in either 9000 × *g* or 105,000 × *g* supernatant fractions obtained from saliva followed by staining for aldehyde dehydrogenase activity (benzaldehyde, octanal, and acetaldehyde as substrates and NAD^+ as cofactor) suggested the presence of a single aldehyde dehydrogenase, one that exhibited an electrophoretic mobility similar, if not identical, to that exhibited by stomach mucosa ALDH-3 (data not shown). Thus, the salivary enzyme was purified and a direct comparison of its physical and kinetic properties with those of purified stomach mucosa ALDH-3 was made.

The apparently pure salivary aldehyde dehydrogenase exhibited a specific activity of 31,000 mIU/mg protein (Table 1), and isoelectric point values characteristic of ALDH-3 (reviewed in Refs. 22 and 25), although they were not exactly identical to those exhibited by stomach mucosa ALDH-3 (Fig. 1). While always falling in the pI range of 5.7–6.4, the exact banding pattern of ALDH-3 seems to inexplicably vary somewhat with the tissue of origin (reviewed in Refs. 22 and 25). As judged by both nondenaturing linear gradient PAGE and gel permeation chromatography on Sephacryl S-200, its relative native molecular weight was 110,000 (data not shown). Antistomach mucosa ALDH-3 IgY recognized the native (data not shown) as well as the denatured (Fig. 2) enzyme, and the relative subunit molecular weight was 54,500. As judged by K_m values, the salivary aldehyde dehydrogenase much preferred benzaldehyde to acetaldehyde as a substrate, and NAD^+ to $NADP^+$ as a cofactor (Table 2). It was only partially inhibited (<30%) by a high concentration of disulfiram (50 μM) and was heat labile, *i.e.*, catalytic activity was completely lost in less than 10 min when it was incubated at 56°C (data not presented). Like all known aldehyde dehydrogenases, the purified salivary enzyme also exhibited esterolytic activity (8390 mIU/mg protein). Each of these physical and catalytic characteristics are essentially identical to those exhibited by stomach mucosa ALDH-3 (22).

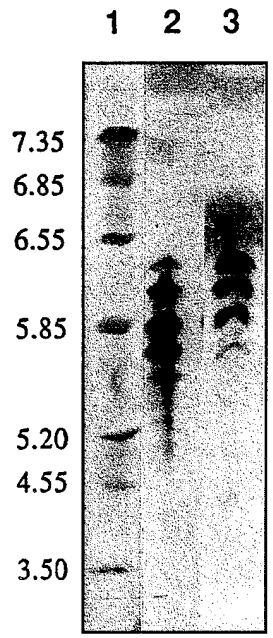

Fig. 1 Isoelectric focusing of the aldehyde dehydrogenase purified from human saliva. Purification and isoelectric focusing of the aldehyde dehydrogenase present in human saliva were as described in "Materials and Methods." Subjected to isoelectric focusing were isoelectric point standards (*Lane 1*), and amounts of purified stomach mucosa ALDH-3 (*Lane 2*) and the aldehyde dehydrogenase purified from human saliva (*Lane 3*) sufficient to generate approximately 10–15 nmol NADH/min (as determined by spectrophotometric assay) when benzaldehyde (4 mM) and NAD^+ (1 mM) were used as substrate and cofactor, respectively. *Lane 1*, stained with Coomassie brilliant blue R-250 for the presence of proteins. *Lanes 2* and *3*, stained for aldehyde dehydrogenase activity as described in "Materials and Methods"; benzaldehyde (4 mM) and NAD^+ (4 mM) were used as the substrate and cofactor, respectively.

Like the stomach mucosa ALDH-3, the salivary ALDH-3 only poorly catalyzed the oxidation of aldophosphamide to carboxyphosphamide (Table 3). This characteristic distinguishes the ALDH-3 present in stomach mucosa and saliva from the ALDH-3s that are present in the two human tumor cell lines that have been, thus far, looked at, namely, MCF-7 breast adenocarcinoma and colon C carcinoma, in that those in the latter catalyze the reaction at measurably greater rates (2, 3, 22, 26–28).

Among individuals, salivary ALDH-3 levels varied widely (Fig. 3). Large interindividual variations in salivary glutathione *S*-transferase and DT-diaphorase levels were also observed (Fig. 3). Indicative of some sort of coordinated regulation of the expression/secretion of these enzymes was the fact that the relative amounts of each in any given saliva sample appeared to be directly related (Fig. 4).

Interindividual variability in the salivary levels of these enzymes could not be attributed to gender, race, ethnicity, alcohol consumption, the use of tobacco, or the consumption of animal products. However, it did appear to be related to coffee consumption since the salivary levels of all three enzymes were, on average, substantially higher in individuals who regularly consumed this beverage (at least 150 ml daily) when compared

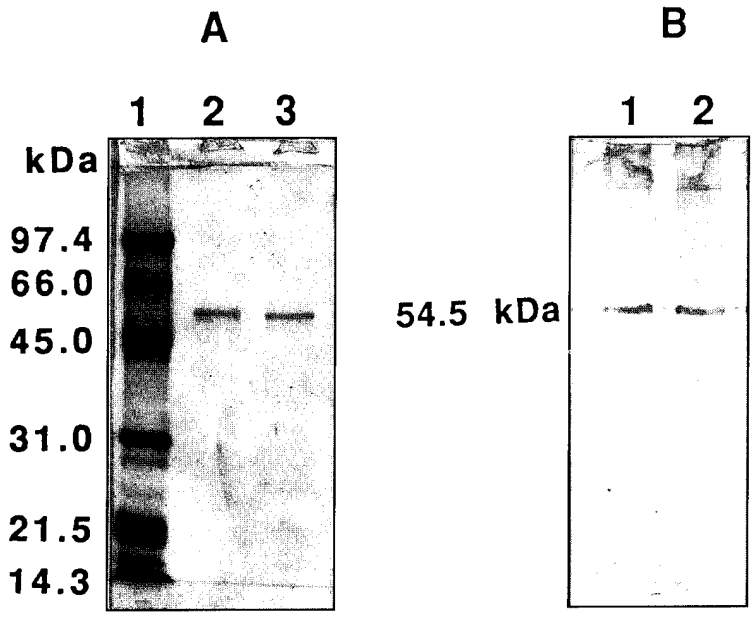

Fig. 2 Subunit molecular weight of the aldehyde dehydrogenase purified from human saliva as determined by SDS-PAGE and recognition of the denatured enzyme by antistomach mucosa ALDH-3 IgY. Purification and SDS-PAGE of the aldehyde dehydrogenase present in human saliva were as described in "Materials and Methods." *A*, subjected to SDS-PAGE were molecular weight markers (*Lane 1*) and 3 μg each of purified stomach mucosa ALDH-3 (*Lane 2*) and the aldehyde dehydrogenase purified from human saliva (*Lane 3*). Molecular weight markers were lysozyme (14.3 kDa), trypsin inhibitor (21.5 kDa), carbonic anhydrase (31 kDa), ovalbumin (45 kDa), BSA monomer (66 kDa), and phosphorylase *b* (97.4 kDa). Proteins in *each lane* were visualized by staining with Coomassie brilliant blue R-250. A plot of log M_r *versus* mobility was used to estimate subunit molecular weights. *B*, purified stomach mucosa ALDH-3 and the aldehyde dehydrogenase purified from human saliva (3 μg each) were submitted to SDS-PAGE and then electrotransferred onto an Immobilon-PVDF transfer membrane. The membrane was then probed with antistomach mucosa ALDH-3 IgY as described in "Materials and Methods" to visualize stomach mucosa ALDH-3 (*Lane 1*) and the aldehyde dehydrogenase purified from human saliva (*Lane 2*).

Table 2 Substrate and cofactor preferences of the aldehyde dehydrogenase purified from human saliva

		K_m (μM)	
Aldehyde (mM)	Cofactor (mM)	Saliva[a]	Stomach Muscosa[b]
Benzaldehyde (0.05–4)	NAD^+ (1)	465	505
	$NADP^+$ (4)	463	486
Acetaldehyde (25–200)	NAD^+ (1)	85,000	80,000
	$NADP^+$ (4)	85,000	81,000
Benzaldehyde (4)	NAD^+ (0.01–1)	40	54
	$NADP^+$ (0.1–4)	1,250	1,000

[a] Each value is the mean of three determinations.
[b] Values for purified stomach mucosa ALDH-3 are from a previous publication (22); they are included here for comparative purposes.

Table 3 Catalysis of aldophosphamide and benzaldehyde oxidation by the aldehyde dehydrogenase purified from human saliva: relative rates[a]

Source	nmol Aldophosphamide oxidized/min/mg (1000) / nmol Benzaldehyde oxidized/min/mg
Saliva	0.32
Stomach mucosa[b]	0.29

[a] Aldehyde dehydrogenase activity was quantified as described in "Materials and Methods"; aldophosphamide (160 μM) or benzaldehyde (4 mM) was the substrate, and NAD^+ (1 mM) was the cofactor.
[b] The value for purified stomach mucosa ALDH-3 is from a previous publication (27); it is included here for comparative purposes.

to the average salivary levels of these enzymes in individuals who did not drink coffee at all (Figs. 3 and 4).

Immunoblot analysis (Fig. 5) confirmed that the level of ALDH-3 was indeed relatively elevated in the saliva of individuals who regularly drank relatively large amounts of coffee, and revealed that salivary levels of glutathione *S*-transferases α, μ, and π were each relatively elevated in such individuals.

Roasted coffee beans are a rich source of catechol and other known inducers, *e.g.*, hydroquinone, of these enzymes (19). Thus, it seemed likely that the relatively elevated levels of salivary ALDH-3, glutathione *S*-transferase, and DT-diaphorase activities that we observed in individuals who consumed relatively large amounts of coffee on a daily basis was, in fact, brought about by an agent(s) present in the coffee, most probably largely catechol and/or hydroquinone, that coordinately induced the expression/secretion of these enzymes.

To further test this notion, salivary ALDH-3, glutathione *S*-transferase, and DT-diaphorase activities were quantified in a volunteer who alternately went without drinking coffee for several days and drinking relatively large amounts of the beverage for several days. The results of the experiment fully supported the notion (Fig. 6). Again, immunoblot analysis (Fig. 7) confirmed that the levels of ALDH-3 were relatively

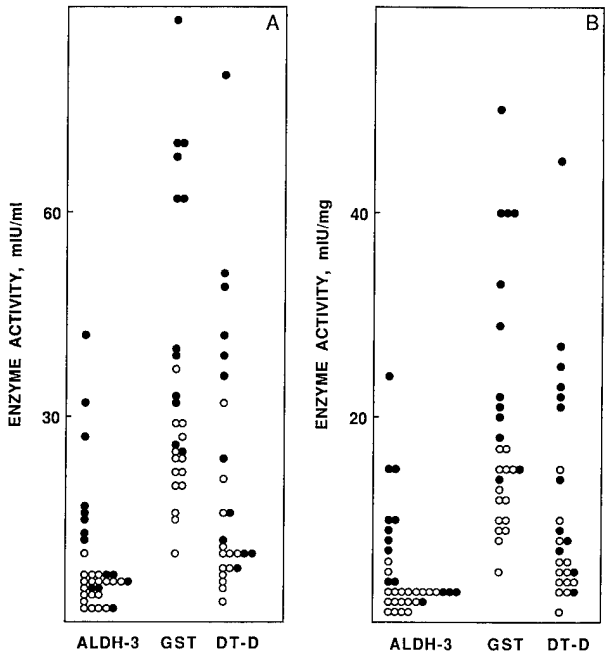

Fig. 3 ALDH-3, glutathione *S*-transferase, and DT-diaphorase levels in human saliva. Collection and processing of saliva and determinations of protein concentrations and enzyme activities were as described in "Materials and Methods." Benzaldehyde (4 mM) and $NADP^+$ (4 mM) were used as substrate and cofactor, respectively, to quantify ALDH-3 activity. Reduced glutathione (5 mM) and 1-chloro-2,4-dinitrobenzene (1 mM) were used to quantify glutathione *S*-transferase (*GST*) activity. 2,6-Dichlorophenol-indophenol (40 μM), NADH (160 μM), and dicumarol (10 μM) were used as substrate, cofactor, and inhibitor, respectively, to quantify DT-diaphorase (*DT-D*) activity. Points are the mean of duplicate determinations made on single samples (3–5 ml) collected from each donor. Enzyme activity was normalized for salivary volume (*A*) and salivary protein (*B*). Overall mean ± SD values were 9 ± 9 mIU/ml saliva and 5 ± 5 mIU/mg salivary 9000 × *g* supernatant protein for ALDH-3, 36 ± 21 mIU/ml saliva and 20 ± 12 mIU/mg salivary 9000 × *g* supernatant protein for glutathione *S*-transferase, and 21 ± 19 mIU/ml saliva and 11 ± 11 mIU/mg salivary 9000 × *g* supernatant protein for DT-diaphorase. ●, subjects who consumed at least 150 ml of coffee daily; mean ± SD values were 15 ± 12 mIU/ml saliva and 8 ± 6 mIU/mg salivary 9000 × *g* supernatant protein for ALDH-3, 51 ± 21 mIU/ml saliva and 28 ± 12 mIU/mg salivary 9000 × *g* supernatant protein for glutathione *S*-transferase, and 31 ± 22 mIU/ml saliva and 17 ± 12 mIU/mg salivary 9000 × *g* supernatant protein for DT-diaphorase. ○, subjects who did not drink coffee at all; mean ± SD values were 5 ± 2 mIU/ml saliva and 3 ± 1 mIU/mg salivary 9000 × *g* supernatant protein for ALDH-3, 23 ± 7 mIU/ml saliva and 12 ± 3 mIU/mg salivary 9000 × *g* supernatant protein for glutathione *S*-transferase, and 11 ± 8 mIU/ml saliva and 6 ± 4 mIU/mg salivary 9000 × *g* supernatant protein for DT-diaphorase. In all cases, salivary enzyme activities of coffee-drinking volunteers were significantly ($P \leq 0.005$) greater than were those of volunteers who did not drink coffee.

elevated in salivary samples taken during the periods of high coffee consumption, and revealed that salivary levels of glutathione *S*-transferases α, μ, and π were each relatively elevated during these periods.

Numerous vegetables, *e.g.*, crucifers such as broccoli, are known to coordinately induce glutathione *S*-transferase and DT-diaphorase activities in various models (5, 6). Furthermore, serum levels of glutathione *S*-transferase α were elevated in subjects who had consumed relatively large amounts of brussels

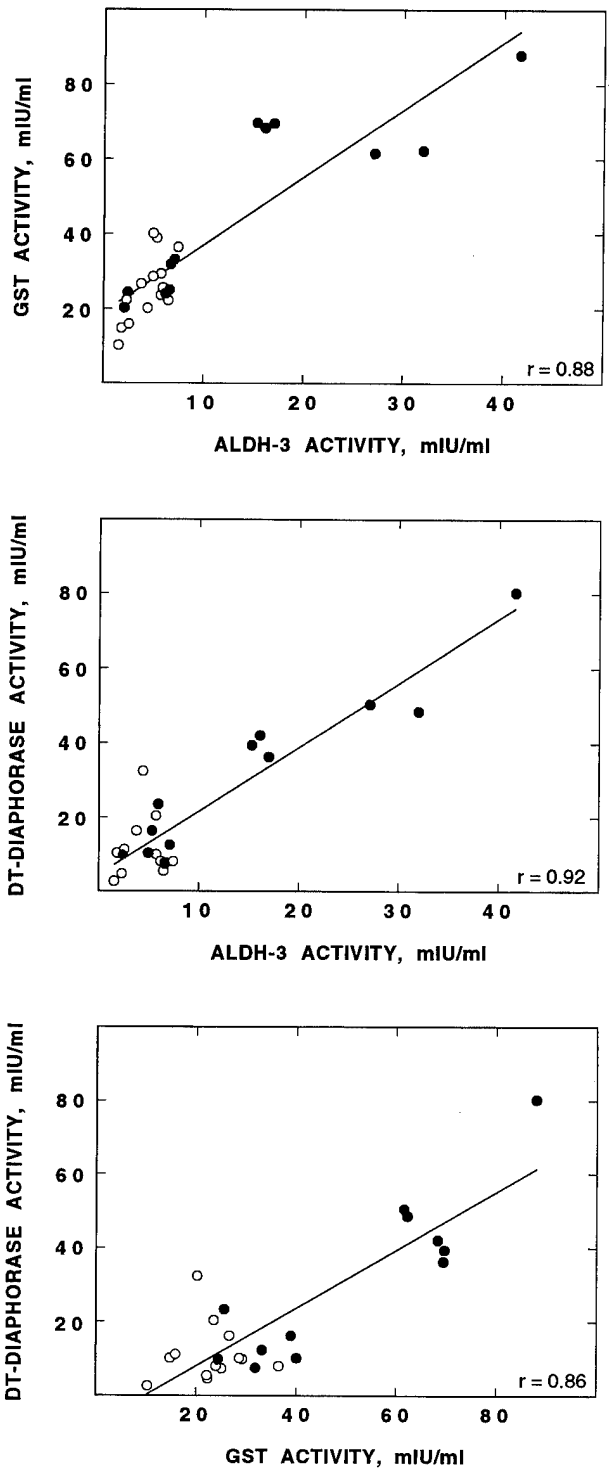

Fig. 4 ALDH-3, glutathione *S*-transferase (*GST*), and DT-diaphorase levels in human saliva. In the investigation presented in Fig. 3, one or more of the three enzyme activities of interest were quantified in single samples obtained from each of 33 subjects; all three were quantified in 25 subjects. Correlations between the magnitudes of enzyme activities in the latter are shown here. ●, subjects who consumed at least 150 ml of coffee daily. ○, subjects who did not drink coffee at all.

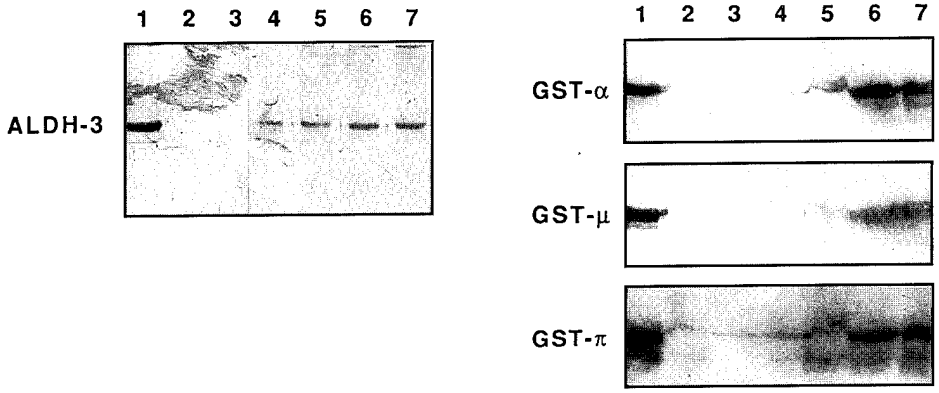

Fig. 5 Immunoblot analysis of six human saliva samples for the physical presence of ALDH-3 and glutathione *S*-transferases α, μ, and π. Three μg of purified stomach mucosa ALDH-3 (*left panel, Lane 1*), 1 μg each of purified human glutathione *S*-transferases (*GST*) α, μ, and π (*right panels, Lane 1*), and equal amounts of processed saliva samples [*left panel*, relevant protein pools (15–20 μg) recovered from 9000 × *g* saliva supernatant fractions (1.5 ml) subjected to reactive blue 2-Sepharose CL-6B affinity chromatography; *right panels*, aliquots (200 μg protein) of 9000 × *g* saliva supernatant fractions] obtained from each of three subjects who did not drink coffee (*Lanes 2–4*) and three subjects who consumed at least 600 ml of coffee daily (*Lanes 5–7*) were first subjected to SDS-PAGE. Proteins thus resolved were then electrotransferred onto Immobilon-PVDF transfer membranes, after which the membranes were probed with antibodies against stomach mucosa ALDH-3 and human glutathione *S*-transferases α, μ, and π as described in "Materials and Methods." Aldehyde dehydrogenase activities (4 mM benzaldehyde, 4 mM $NADP^+$) in the original saliva samples were 2, 3, 7, 27, 32, and 42 mIU/ml (*left panel, Lanes 2–7*, respectively). Glutathione *S*-transferase activities in the original saliva samples were 22, 16, 36, 62, 62, and 88 mIU/ml (*right panels, Lanes 2–7*, respectively).

sprouts (29). Not unexpectedly then, salivary ALDH-3, glutathione *S*-transferase, and DT-diaphorase activities were relatively high during a period of high broccoli consumption by a volunteer, whereas they were much lower before and after this period (Fig. 8). Once again, immunoblot analysis (Fig. 9) confirmed that the levels of ALDH-3 were relatively elevated during the period of high broccoli consumption, and revealed that salivary levels of glutathione *S*-transferases α, μ, and π were each relatively elevated during this period.

The origin of salivary ALDH-3, the glutathione *S*-transferases, and DT-diaphorase is not known. One possibility is that these enzymes are synthesized and then secreted into the saliva by one or more of the various salivary glands. Supporting this scenario is the observation that each of these enzymes is found in these glands (30–32).[5] Another possibility is that they are synthesized in more distant organs/tissues, *e.g.*, liver, and that they ultimately arrive in the saliva via the circulatory system. The presence of glutathione *S*-transferase α and glutathione *S*-transferase activity in human plasma has been reported (29, 33). Both glutathione *S*-transferase and DT-diaphorase activities are reportedly present in rodent serum (15). We were unable to detect the presence of either ALDH-3 or DT-diaphorase in plasma samples, even when such samples were taken from subjects who had consumed large amounts of coffee (Table 4). Prochaska and Fernandes (15) were also unable to detect any DT-diaphorase activity in human serum. On the other hand, relatively low-level glutathione *S*-transferase activity was present in plasma samples from donors who regularly consumed large amounts of coffee as well as in those obtained from donors who did not drink coffee at all. Plasma glutathione *S*-transferase levels were higher in the former and roughly reflected the relative levels of this enzyme in the saliva.

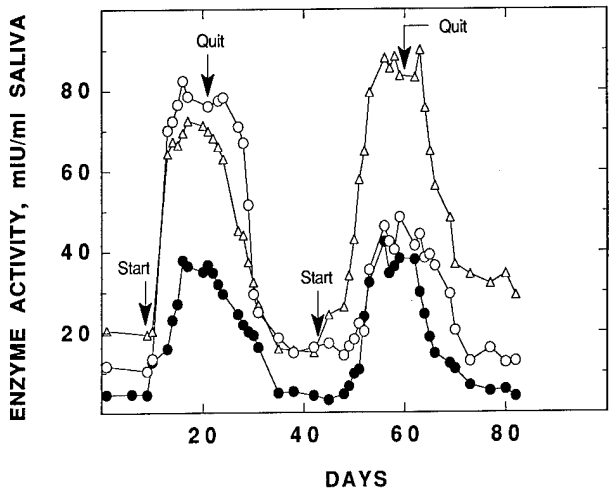

Fig. 6 Effect of drinking coffee on the levels of ALDH-3, glutathione *S*-transferase, and DT-diaphorase activities in human saliva. ALDH-3 (●), glutathione *S*-transferase (△), and DT-diaphorase (○) activities in the saliva of a healthy male subject, age 27 years, who had not consumed coffee for at least 3 weeks prior to the beginning of this experiment, and who partook of a "normal" diet and did not partake of alcohol, carbonated beverages, tobacco, or prescription medications during the experiment, were quantified for 9 days to establish basal levels of these enzyme activities. The subject then drank 1–1.2 liters of regular black coffee/day for 12 consecutive days (days 9–20), after which he completely stopped consuming coffee for a period of 22 days (days 21–42). The sequence of drinking coffee (days 43–59) and then stopping (day 60 and beyond) was then repeated. Collection and processing of saliva, and quantification of ALDH-3 (4 mM benzaldehyde, 4 mM $NADP^+$), glutathione *S*-transferase, and DT-diaphorase activities were as described in "Materials and Methods."

DISCUSSION

Harada *et al.* (17) were the first to report the presence of an aldehyde dehydrogenase in human saliva. Upon characterizing

[5] L. Sreerama and N. E. Sladek, manuscript in preparation.

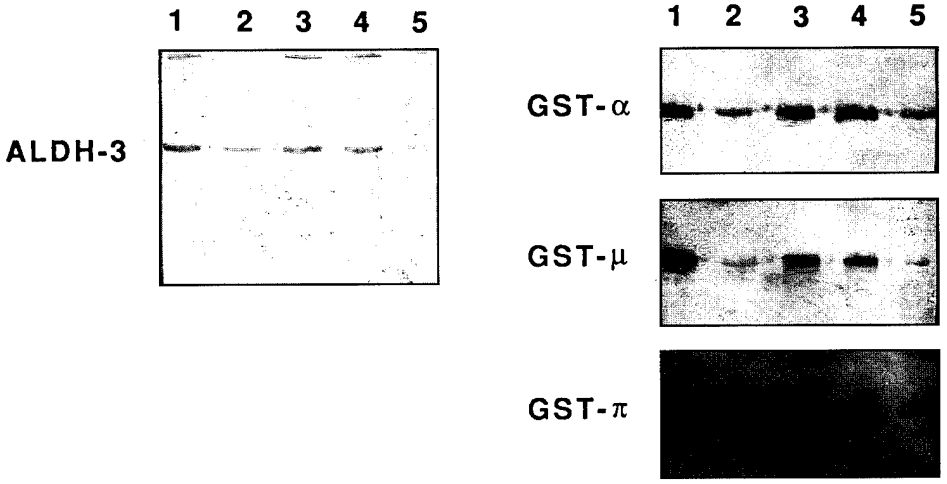

Fig. 7 Immunoblot analysis of human saliva samples obtained at various time points during the course of the experiment described in the legend to Fig. 6 for the physical presence of ALDH-3 and glutathione *S*-transferases α, μ, and π. Three μg of purified stomach mucosa ALDH-3 (*left panel, Lane 1*), 1 μg each of purified human glutathione *S*-transferases (*GST*) α, μ, and π (*right panels, Lane 1*), and equal amounts of processed (exactly as detailed in the legend to Fig. 5) saliva samples obtained on days 1 (*Lane 2*), 17 (*Lane 3*), 22 (*Lane 4*), and 42 (*Lane 5*) of the experiment described in the legend to Fig. 6 were first subjected to SDS-PAGE. Proteins thus resolved were then electrotransferred onto Immobilon-PVDF transfer membranes, after which the membranes were probed with antibodies against stomach mucosa ALDH-3 and human glutathione *S*-transferases α, μ, and π as described in "Materials and Methods."

the enzyme [subunit M_r 48,000; failure of antiserum raised against the salivary enzyme to recognize authentic ALDH-1, ALDH-2, ALDH-3, or ALDH-4 (glutamic γ-semialdehyde dehydrogenase); a K_m of 106 μM when acetaldehyde was the substrate], they declared it to be a unique, theretofore unidentified, aldehyde dehydrogenase and gave it the name ALDH-V (18). Herein, we, too, report the presence of an aldehyde dehydrogenase in human salivary samples. However, the enzyme that we found exhibited physical and catalytic properties essentially identical to those exhibited by stomach mucosa ALDH-3 (22). It was the only aldehyde dehydrogenase that we could find in this fluid. The reason for the discrepancy between our laboratory and that of Harada *et al.* (18) is unclear. One, albeit remote, possibility is that the subject(s) from whom they obtained the salivary sample(s) happened to be one(s) expressing a variant ALDH-3.

Unknown is the purpose that the presence of ALDH-3, the glutathione *S*-transferases, and DT-diaphorase in the saliva serves. Saliva is viewed primarily as a digestive secretion. Large volumes of it are secreted (600 ml/day in humans) by the salivary glands (34). ALDH-3, the glutathione *S*-transferases, and DT-diaphorase are known to catalyze the detoxification of various xenobiotics (reviewed in Refs. 4, 35, and 36). Toxic, and potentially toxic, xenobiotics, many of which are substrates for these enzymes, are abundantly present in the environment/diet (4, 37–39) and/or can be generated by microflora in the oral cavity (40, 41). Thus, it is tempting to speculate that these enzymes are present in the saliva, in cells lining the alimentary canal, and in cells lining other tissues that constitute so-called "ports of entry," *e.g.*, lungs, for the purpose of detoxifying such agents, although, in the case of certain xenobiotics, toxification is actually effected (42). Consistent with this notion, ALDH-3, glutathione *S*-transferase, and DT-diaphorase activities are relatively high in the lung and in the tissues that constitute the alimentary canal (reviewed in Refs. 1, 43, and 44). However, the

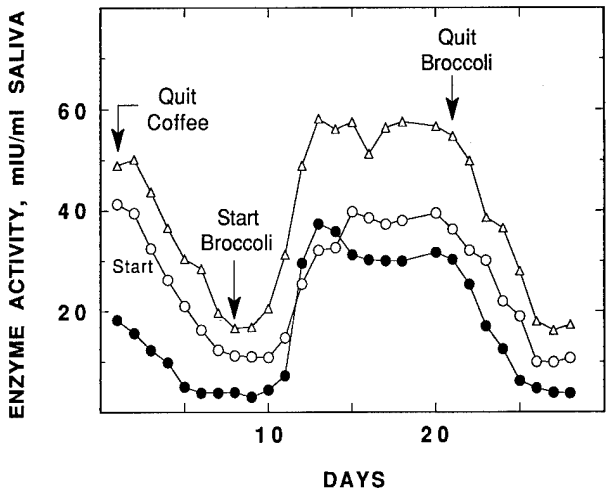

Fig. 8 Effect of eating broccoli on the levels of ALDH-3, glutathione *S*-transferase, and DT-diaphorase activities in human saliva. ALDH-3 (●), glutathione *S*-transferase (△), and DT-diaphorase (○) activities in the saliva of a healthy female subject, age 28 years, who quit consuming coffee at the beginning of this experiment, and who partook of a "normal" diet and did not partake of coffee, alcohol, carbonated beverages, tobacco, or prescription medications during the experiment, were quantified for 8 days to establish basal levels of these enzyme activities. The subject then consumed 300 g of microwave oven-cooked (power output of 650 W for 2 min) broccoli/day for 12 days (days 8–20), after which she completely stopped consuming broccoli (day 21 and thereafter). Collection and processing of saliva, and quantification of ALDH-3, glutathione *S*-transferase, and DT-diaphorase activities were as described in "Materials and Methods."

salivary aspect of this notion is problematic. First, glutathione, NAD(P)H, and $NAD(P)^+$ would have to be present in the saliva at concentrations sufficient to enable the glutathione *S*-transferases, DT-diaphorase, and ALDH-3, respectively, to catalyze

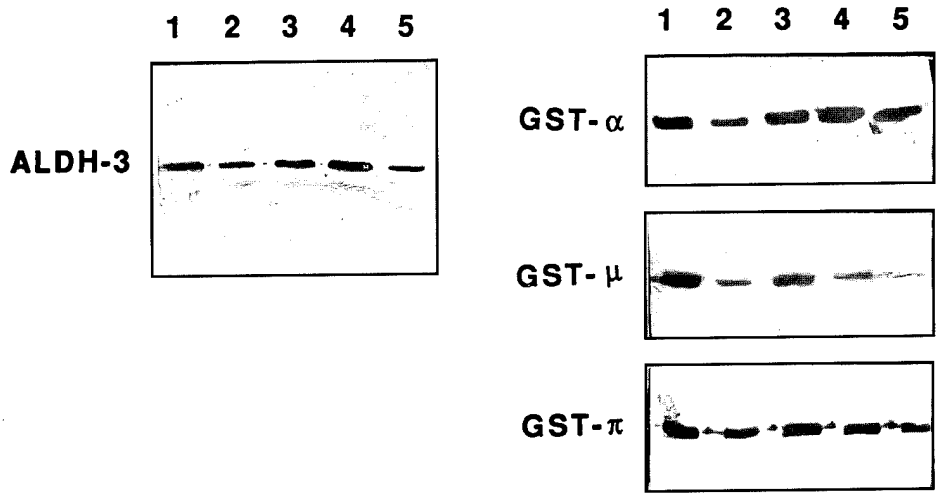

Fig. 9 Immunoblot analysis of human saliva samples obtained at various time points during the course of the experiment described in the legend to Fig. 8 for the physical presence of ALDH-3 and glutathione *S*-transferases α, μ, and π. Three μg of purified stomach mucosa ALDH-3 (*left panel, Lane 1*), 1 μg each of purified human glutathione *S*-transferases (*GST*) α, μ, and π (*right panels, Lane 1*), and equal amounts of processed (exactly as detailed in the legend to Fig. 5) saliva samples obtained on days 7 (*Lane 2*), 13 (*Lane 3*), 21 (*Lane 4*), and 28 (*Lane 5*) of the experiment described in the legend to Fig. 8 were first subjected to SDS-PAGE. Proteins thus resolved were then electrotransferred onto Immobilon-PVDF transfer membranes, after which the membranes were probed with antibodies against stomach mucosa ALDH-3 and human glutathione *S*-transferases α, μ, and π as described in "Materials and Methods."

Table 4 ALDH-3, glutathione *S*-transferase, and DT-diaphorase activities in matching plasma and saliva samples obtained from human male volunteers[a]

	Enzyme activity (mIU/ml)			
	Saliva			Plasma[c]
Subject[b]	ALDH-3	Glutathione *S*-transferase	DT-D	Glutathione *S*-transferase
1	2.6	16.7	14.0	6.4
2	3.5	28.0	4.6	7.2
3	5.0	16.0	8.0	9.0
4	17.0	71.0	36.0	10.5
5	35.5	76.0	39.6	11.0

[a] Collection of blood and saliva from human donors, further processing of these fluids, and determination of ALDH-3, glutathione *S*-transferase, and DT-diaphorase (DT-D) activities were as described in "Materials and Methods." Each value is the mean of duplicate determinations made on each sample.

[b] Subjects 1–3 did not drink coffee at all; subjects 4 and 5 consumed at least 500 ml of coffee daily.

[c] Measurable levels of ALDH-3 and DT-diaphorase activities were not found in any of the plasma samples. The minimum amounts of ALDH-3, glutathione *S*-transferase, and DT-diaphorase activities detectable by the spectrophotometric assay used herein were 1, 1, and 2 mIU/ml saliva or plasma, respectively. In a further attempt to detect the presence of ALDH-3 in the plasma, 8–10-ml blood samples were taken from the same individuals and processed as before. The plasma samples thus obtained were subjected to reactive blue 2-Sepharose CL-6B affinity chromatography, and the resultant preparations were concentrated and submitted to SDS-PAGE/immunoblot analysis as well as spectrophotometric assay for catalytic activity; no ALDH-3 was found.

their respective detoxifying reactions at meaningful rates, although the cofactor would not be required for ALDH-3-catalyzed esterolytic detoxification/digestion. Salivary levels of glutathione are reportedly 1–3 μM (45); those of NAD^+, $NADP^+$, NADH, and NADPH are unknown. Second, enzyme-catalyzed detoxification in the oral cavity would have to be effected very quickly since (*a*) any food that is placed therein is ordinarily swallowed in a matter of seconds, (*b*) none of these enzymes are catalytically active at the low pH (~2.0) that ordinarily prevails in the stomach contents,[6] and (*c*) none are catalytically active after a brief exposure (30 min) to a pH of 2.0,[6] *i.e.*, they would not be active upon reaching the slightly alkaline pH that ordinarily prevails in the contents of the upper small intestine.

It is unclear at this point as to which component(s) of coffee/broccoli is(are) responsible for the increased salivary levels of ALDH-3, glutathione *S*-transferases, and DT-diaphorase, but there are some very likely candidates. Thus, certain constituents of green coffee beans, *e.g.*, kahweol palmitate and cafestol palmitate, have been shown to induce glutathione *S*-transferase activity in mouse liver and intestine (46), and roasted coffee beans are known to be rich in catechol and hydroquinone (19), agents that have been shown to induce ALDH-3, glutathione *S*-transferase, and DT-diaphorase, but not cytochrome P450 IA1 activities (thus, they are, by definition, monofunctional inducers) in cultured human breast and colon tumor cells (3). Sulforaphane is known to be present in broccoli (6) and to induce DT-diaphorase and glutathione *S*-transferase, but not cytochrome P450s IA1 and IA2, activities (thus, again by definition, a monofunctional inducer) in cultured murine hepatoma cells and mouse tissues, *e.g.*, liver, stomach, and intestine (6).

It is now well-established that certain dietary constituents and man-made chemicals prevent experimental carcinogenesis (reviewed in Refs. 7–9). The hope, even expectation, is that they

[6] L. Sreerama and N. E. Sladek, unpublished observations.

will do so in humans. Indeed, several epidemiological studies support this notion (reviewed in Refs. 10 and 47), and Phase I clinical trials in that regard have already been initiated (10–12). Many of the substances that act as so-called chemopreventive agents in experimental carcinogenesis also coordinately induce ALDH-3, glutathione *S*-transferase, and DT-diaphorase activities (3, 6, 15). Glutathione *S*-transferases and DT-diaphorase are known to catalyze the detoxification of a number of known carcinogens (reviewed in Refs. 4 and 35). Thus, it has been suggested that in those cases where a substance induces these enzymes and is chemopreventive, detoxification of potential carcinogens by the induced enzymes is likely to be the causal mechanism of chemoprevention (reviewed in Refs. 4, 7–9, and 35). ALDH-3 has not been studied in that regard, but it is known to catalyze the detoxification of the antineoplastic agents collectively known as oxazaphosphorines, *e.g.*, cyclophosphamide and ifosfamide (1–3, 22, 26–28, 48–50). Oxazaphosphorines are known human carcinogens (51).

Unknown is whether salivary levels of ALDH-3, glutathione *S*-transferases, and DT-diaphorase reflect tissue, *e.g.*, colon epithelium, levels of these enzymes, but that would seem likely. Should that prove to be the case, the measurement of salivary levels of one or more of these enzymes (only one would have to be measured since they are coordinately induced by both monofunctional and bifunctional inducers) in Phase I clinical trials would be a relatively easy and noninvasive way of gaining insight as to the dosage schedules to be used in subsequent trials in those cases where a suspected chemopreventive agent is thought to bring about chemoprevention by inducing the expression of these enzymes. Such measurements could also be useful as an indicator when exposure to environmental/industrial/dietary agents, some of which are known carcinogens, *e.g.*, polycyclic aromatic hydrocarbons such as 3-methylcholanthrene and 3,4-benzpyrene, that induce these enzymes is suspected. They could also be used to preliminarily evaluate the chemopreventive potential of various diets and drugs, although agents effecting a chemoprotective action by another mechanism would not be identified by such measurements. Coordinated induction of ALDH-3, glutathione *S*-transferases, and DT-diaphorase is brought about by both monofunctional and bifunctional inducers (1–3, 22). Which was causative in the case of any given diet could not be ascertained by measuring salivary enzyme activity, but the distinction would only be important if chemoprevention was effected by one of the enzymes only induced by bifunctional inducers, *e.g.*, cytochrome P450s IA1 and IA2.[7]

Given that elevated levels of ALDH-3, glutathione *S*-transferases, and DT-diaphorase in the saliva directly reflect the chemoprotective potential of an agent, our observations with coffee and broccoli predict that continuous ingestion of either will effect, in fact, a chemopreventive action. A review of epidemiological studies already published suggests that continuous ingestion of coffee reduces the risk of colon cancer occurrence, increases the risk of endometrial cancer, leukemia, and ovarian cancer occurrence, and does not change the risk of breast, bladder, kidney, pancreatic, and prostate cancer occurrence (19). Epidemiological studies already published suggest that consumption of broccoli by humans reduces the risk of colorectal and lung cancer occurrence (52–54).

ALDH-3 catalyzes the detoxification of cyclophosphamide and other oxazaphosphorines (1–3, 22, 26–28, 48–50), and the glutathione *S*-transferases catalyze the detoxification of certain alkylating and other chemotherapeutic agents, *e.g.*, chlorambucil, melphalan, and cisplatin (reviewed in Ref. 55). Conversely, DT-diaphorase catalyzes the activation of certain antineoplastic drugs, *e.g.*, mitomycin C and the indoloquinone EO9 (reviewed in Ref. 42). It follows, then, that the coordinated induction of these enzymes by either monofunctional or bifunctional inducers would decrease the therapeutic effectiveness of some antineoplastic agents and increase that of others (discussed in detail in Refs. 2 and 3). Thus, given that salivary levels of these enzymes reflect tissue levels of them, monitoring salivary ALDH-3, glutathione *S*-transferase, and/or DT-diaphorase levels could be of value in the optimization of chemotherapeutic protocols (choice of drug, dose) as well. Moreover, deliberate induction of these enzymes prior to chemotherapy could be of therapeutic benefit in certain scenarios. Finally, maximum induction of these enzymes occurs rapidly, only a few days after first introducing the inducer, and enzyme levels return to basal levels rapidly, again within a matter of days after the inducer is removed (Refs. 2 and 3 and the present study). It is possible then that a drug may be effective (ineffective) at one point in time, and, with a relevant change in diet, may be ineffective (effective) a few months or even weeks later, *i.e.*, tumor sensitivity (resistance) to the drug would (appear to) be transient.

[7] In contrast to ALDH-3, glutathione *S*-transferases α, μ, and π, and DT-diaphorase [NAD(P)H:quinone oxidoreductase; NQO_1], all of which are cytosolic enzymes, cytochrome P450s IA1 and IA2 are membrane embedded. Thus, the latter would not be expected to be secreted into the saliva and, indeed, they do not appear to be present in it.[6]

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